MECHANISMS OF ENVIRONMENTAL TOBACCO SMOKE AND BENZO[A]PYRENE INDUCED CARDIOVASCULAR INJURY AND THE PROTECTIVE ROLE OF RESVERATROL

A thesis submitted to the

College of Graduate Studies and Research

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

through the Toxicology Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan

By

Ahmad Nizar Al-Dissi

December 2010

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirement for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor who supervised my thesis work:

Dr. Lynn Weber
Department of Veterinary Biomedical Sciences

In her absence, permission may be granted by the director of the Toxicology Center at the University of Saskatchewan.

It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests of permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Director, Toxicology Center
University of Saskatchewan
52 Campus Drive
Saskatoon, Saskatchewan S7N 5B4

ABSTRACT

Despite extensive research, the mechanisms behind cardiovascular effects of subchronic environmental tobacco smoke (ETS) remain unclear, but may be related to ETS-induced inflammation and oxidative stress. Additionally, the protective role of resveratrol (RES), a natural antioxidant available in red grapes, is controversial. We hypothesized that the polycyclic aromatic hydrocarbon (PAH) component of ETS is responsible for causing adverse cardiovascular effects. We also hypothesized that the administration of RES is protective against the adverse cardiovascular effects of ETS. In order to address these hypotheses, male juvenile pigs (4-weeks old) were exposed to ETS or ambient air for 28 consecutive days (1 hr/day) and effects compared to 7 days of i.v. injection of the PAH, benzo-a-pyrene (BAP; 5 mg/kg daily). In another experiment, pigs were sham-exposed or ETS-exposed, with or without oral RES treatment (5mg/kg daily). In all experiments, endothelial and left ventricular function were assessed by flow mediated dilation (FMD), and echocardiography, respectively, while blood pressure was evaluated by oscillometry. At the termination of each experiment, serum nitrotyrosine, total nitrate/nitrite (NOx) and C-reactive protein (CRP) were measured as well as hepatic and pulmonary ethoxyresorufin-o-deethylase (EROD) activity to indicate cytochrome P450 1A1 (CYP1A1) expression. Finally, the correlation between pulmonary inflammation and adverse cardiovascular effects was investigated by measuring total and differential white blood cell (WBC) count as well as leukocyte elastase activity in bronchoalveolar lavage fluid at the termination of each experiment. ETS exposure, but not BAP treatment, resulted in a significant impairment of FMD ($P<0.0001$) and increased left ventricular end diastolic volume ($P=0.0032$). Cotreatment with RES failed to restore the ETS induced impairment of FMD ($P>0.05$). However, a trend pointing to an increase in ejection fraction (EF) was noted ($P=0.072$). ETS, BAP and RES treatments failed to have any effect on blood pressure ($P>0.05$). BAP injection caused a significant increase in serum nitrotyrosine ($P=0.0146$) and CRP ($P=0.012$), but not

serum NOx levels (P>0.05). In contrast, ETS exposure resulted in a significant increase in CRP serum levels (P=0.0092), a trend pointing to increased serum nitrotyrosine (P=0.105), and no change in serum NOx levels (P>0.05). The increased nitrotyrosine and CRP with ETS exposure was not reversed by RES administration (P>0.05). ETS exposure increased EROD activity in the lung (P=0.0093), but not the liver (P=0.12). In contrast, BAP treatment had the opposite effect (lung EROD: P=0.621, liver EROD: P=0.01), while RES administration had no effect (P>0.05). ETS exposure (P=0.0139), but not BAP treatment (P=0.723), resulted in increased WBC count in BAL fluid which was not affected by RES administration (P>0.05). These results show that ETS exposure causes lung inflammation, systemic inflammation, oxidative stress-mediated inactivation of nitric oxide and impaired endothelial function. In contrast, BAP failed to alter endothelial function, downstream of the lung, despite systemic inflammation and increased oxidative stress. Furthermore, RES failed to restore endothelial function, or decrease systemic inflammation and oxidative stress. Taken together, these results suggest either that pulmonary inflammatory responses or pulmonary increases in CYP1A1 activity may be more important links to endothelial dysfunction than systemic inflammation and nitric oxide bioactivity. The beneficial effects of RES by itself are manifested only at the cardiac level by improving the ejection fraction, but the work in this thesis failed to detect any ability of RES to ameliorate ETS cardiovascular effects.

ACKNOWLEDGMENTS

This work could not have been possible without the help of several people. I would like first to thank my research supervisor Dr. Lynn Weber for her guidance and support throughout this project. Her patience and encouragement made it possible for me to get an excellent experience in research. I also would like to thank my committee members Drs. Linda Hiebert, Barry Blakley, Lily Wu, and John Gordon for their support and expertise.

This thesis is dedicated to my family for all their love and support throughout my life, including my parents Nizar and Muna, my wife Rana, my sisters: Abeer, Noor and Sawsan and my brother Sulaiman. I also would like to thank the members of the Weber laboratory as well as fellow students in the toxicology program, including Jordan Marit, Nicole Gentner, Brandie Bugiak, Meghan Goertzen, Jennifer Adolphe, and Lilani Munasinghe.

Finally, the funding and support for this project was provided by a generous grant from the Saskatchewan Health Research Foundation. I was supported by a generous scholarship from the WCVM Interprovincial Graduate Fellowship.

TABLE OF CONTENTS

LIST OF TABLES

Table 5.7: Wall thickness and diameter of arteries after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Luminal diameter (LD), wall thickness (WT) and luminal diameter/wall thickness (LD/WT) of aorta, brachial and coronary arteries were determined by digital morphometric analyses of hematoxylin/eosin-stained sections. Data are expressed as mean ± standard error of the mean. No statistically significant differences were detected using separate two-way analyses of variances with ETS and RES as factors. 101

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ahr: aryl hydrocarbon receptor

ANOVA: analysis of variance

BAL: bronchoalveolar lavage

BAP: benzo[a]pyrene

BH4: tetrahydrobiopterin

CO-Hb: carboxyhemoglobin

cGMP: cyclic guanosine monophosphate

CO: carbon monoxide

COPD: chronic obstructive pulmonary disease

CRP: C-reactive protein

CVD: cardiovascular disease

EDV: end diastolic volume

EF: ejection fraction

eNOS: endothelial nitric oxide synthase

EROD: ethoxyresorufin-o-deethylase

ESV: end systolic volume

ETS: environmental tobacco smoke

FMD: flow mediated dilation

H & E: hematoxylin and eosin

ICAM-1: intercellular adhesion molecule-1

IL-1: interleukin-1

IL-1β : interleukin-1β

IL-6: interleukin-6

LD: luminal diameter

LDL: low density lipoprotein

LDLR: low density lipoprotein receptor

LTB-4: leukotriene B4

MAP: mean arterial pressure

MCP-1: macrophage chemoattractant protein-1

MCSF: macrophage colony stimulating factor

MetHb: methemoglobin

MIP-1: macrophage inflammatory protein-1

MMP-12: matrix metalloproteinase-12

MMP-9: matrix metalloproteinase-9

NO: nitric oxide

NOx: nitrate/nitrite

NAD^+: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

OxyHb: oxyhemoglobin

PAHs: polycyclic aromatic hydrocarbons

RES: resveratrol

ROS: reactive oxygen species

SEM: standard error of the mean

Sir2: sirtuin information regulator 2 found in yeast

SIRT1: mammalian homologue of Sir2

TNF-α: tumour necrosis factor-α

TotalHb: total hemoglobin

VCAM-1: vascular cell adhesion molecule-1

WT: wall thickness

1. Introduction

This study aimed to determine the impact of a long term exposure to environmental tobacco smoke (ETS) on the cardiovascular and pulmonary systems utilizing a swine model. Another goal was to determine the role of polycyclic aromatic hydrocarbons (PAHs) in mediating these effects through the intravenous injection of a specific ETS constituent i.e. benzo[a]pyrene (BAP). The same suite of cardiovascular, pulmonary, biochemical and histological parameters was measured in both studies.

The experimental work in this thesis is divided into two sections, each written in a manuscript format ready for publication. The first manuscript deals with the effects of ETS and BAP on the cardiovascular system, and the second examines the cardiovascular protective role of resveratrol (RES) when co-administered with ETS exposure. The thesis is divided into 7 chapters with the first one being this introduction. Chapter 2 is the literature review, where most recent studies about this topic are cited. Chapter 3 contains the hypotheses and objectives of this thesis. This is followed by chapter 4, containing the first manuscript of this thesis. Chapter 5 contains the 2nd manuscript of this thesis. Chapter 6 contains the overall discussion and conclusion of this thesis. Finally, chapter 7 lists all the references cited in this thesis.

2. Literature review

2.1. Cardiovascular disease (CVD) statistics and risk factors

The term cardiovascular disease (CVD) refers to a number of different disease processes that involve the heart and vasculature, including coronary heart disease, peripheral artery disease and stroke. Cardiovascular disease remains a major cause of death in North America and worldwide. In the United States 2300 Americans die of CVD on a daily basis with coronary heart disease responsible for 1 in every 6 deaths in 2006 (Lloyd-Jones *et al.*). In Canada it is estimated that 1 person dies from heart disease or stroke every 7 minutes. In 2006, CVD accounted for 30% of deaths in Canada (Statistics Canada, 2010).

Several risk factors have been identified for CVD some of which are modifiable while others are not. Among the modifiable risk factors smoking, obesity, decreased activity, high blood cholesterol and glucose, and high blood pressure are most important. Non-modifiable risk factors include genetics, aging and male gender (O'Donnell and Kannel, 1998).

2.2. Cardiovascular disease mechanisms

2.2.1.Atherosclerosis

The main pathophysiological process involved in the development of CVD is atherosclerosis (Kumar V, 2010). Atherosclerosis is a disease affecting medium and large sized arteries characterized by the presence of subintimal thickening known as atheromatous plaques. These arterial plaques are composed of a central core of lipid infiltrated by macrophages and surrounded by a fibrous cap. Atheromatous plaques not only interfere with blood flow but can also result in vascular thrombosis and aneurysm (Crowther, 2005).

Two major theories have been proposed to explain the development of atherosclerosis. The first theory is known as the response to injury hypothesis. According to this theory, the initial event in atherosclerosis development is endothelial injury. Endothelial injury is followed by modulation of growth factors and cytokine production resulting in uncontrolled smooth muscle proliferation (Ross and Glomset, 1973). In contrast, Benditt explained the development of atherosclerotic lesions as benign neoplasms arising from smooth muscles within the vasculature (Benditt and Benditt, 1973). The earliest lesion seen in atherosclerosis is known as fatty streak which is composed of lipid laden macrophages within the subendothelial space. This is followed by the migration of smooth muscles from tunica media into the subintimal space and extracellular matrix production to form the atheromatous plaque. The major mechanisms involved in atherosclerosis include endothelial dysfunction, hemodynamic disturbances, hyperlipidemia and hyperglycemia, and infection (Kumar V, 2010).

2.2.2.Normal endothelial function

Endothelial cells play a key role in the regulation of vascular tone as well as coagulation and inflammation, all of which are implicated in the development of atherosclerosis. The endothelium is responsible for the regulation of cardiovascular tone by generating active mediators including nitric oxide (NO), endothelium-derived relaxing factor, and endothelin (Bellien *et al.*, 2008; Busse and Fleming, 2006; Luscher, 1994). Normally, increased arterial shear stress results in increased intracellular calcium within endothelial cells (Busse and Fleming, 2006). As a result, an enzyme known as endothelial NO synthase (eNOS) is

activated and NO is generated (Busse and Fleming, 2006). After diffusing into smooth muscles, NO activates the enzyme guanylate cyclase which increases the production of cyclic guanosine monophosphate (cGMP). cGMP activates cGMP-dependent protein kinase which mediates vascular smooth muscle relaxation and ultimately results in vasorelaxation (Munzel *et al.*, 2005). Vascular smooth muscles are also capable of proliferation, migration and extracellular matrix deposition. Such functions are physiologically regulated by growth promoters and inhibitors (Rzucidlo, 2009). Promoters include platelet derived growth factor, produced by platelets, endothelial cells, and macrophages, basic fibroblast growth factor, and interleukin-1 (IL-1). Inhibitors include NO and transforming growth factor- β (Rzucidlo, 2009).

By modulating the expression of adhesion molecules on its surface the endothelium plays a role in regulating inflammation (Galkina and Ley, 2007). For example, the endothelium expresses intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) both of which are essential for macrophage migration in atherosclerosis. Intercellular adhesion molecule-1 knockout mice are protected from atherosclerosis (Collins *et al.*, 2000) and mice with reduced VCAM-1 expression have reduced atherosclerotic lesions (Dansky *et al.*, 2001). Nitric oxide also regulates several other processes including platelet activation and coagulation (Radomski *et al.*, 1990). Therefore, the impairment of NO production is involved in the progression of atherosclerosis.

2.2.3.Endothelial dysfunction

Endothelial dysfunction represents an important early step in the pathogenesis of atherosclerosis. Endothelial dysfunction is characterized by endothelial cells becoming proinflammatory and prothrombotic with reduced vasodilator function (Endemann and Schiffrin, 2004). Several disease processes and toxins may contribute to the development of atherosclerosis by causing endothelial dysfunction. Examples include hypertension, hypercholesterolemia, cigarette smoking, and diabetes (Kumar V, 2010). Endothelial injury is usually associated with increased oxidative stress (Schulz *et al.*, 2008). The interaction of reactive oxygen species (ROS), such as superoxide anion, with NO, produced by endothelial cells, results in the formation of peroxynitrite (Schulz *et al.*, 2008). Peroxynitirite can impair vasodilatation by inactivating the enzymes eNOS and prostacyclin synthase (Zou *et al.*, 2002a; Zou and Ullrich, 1996). Peroxynitrite is highly reactive, short lived and forms nitrotyrosine after it interacts with tyrosine residues in proteins. Not only can peroxynitrite uncouple the enzyme eNOS, but it also can turn it into a source of free radicals (Zou *et al.*, 2002b). When uncoupled, eNOS generates superoxide anions by transferring electrons to molecular oxygen instead of L-arginine (Munzel *et al.*, 2005; Schulz *et al.*, 2008). The net result is decreased eNOS activity, decreased NO production, decreased NO bioavailability and increased oxidative stress. Underlying mechanisms for eNOS uncoupling include the depletion of tetrahydrobiopterin (BH4), a cofactor for eNOS production, and L-arginine, the substrate for NO (Mehta *et al.*, 2006).

Ultimately, endothelial injury causes subintimal lipid accumulation and increased expression of adhesion molecules on the surface of endothelial cells. In humans, brachial

artery flow-mediated dilation, measured using high resolution ultrasound, is a noninvasive and broadly applicable method used to evaluate endothelial function (Martin, 2009).

2.2.4.Roles of elevated blood lipids and glucose

Cholesterol and fatty acids are transported in the circulation by many plasma lipoproteins. Low density lipoprotein (LDL) is the principal plasma cholesterol carrier (Crowther, 2005). Low density lipoprotein is recognized and internalized by the LDL receptor (LDLR), which is ubiquitously expressed (Miller *et al.*, 2010). In situations with increased oxidative stress LDL is modified to an oxidized form i.e. oxLDL (Li and Mehta, 2005). Endothelial cells internalize oxLDL through LOX-1 receptors (Sawamura *et al.*, 1997). Additionally, LDL can be modified by enzymes and free radicals within the subintimal space to form oxLDL. Then oxLDL is taken up by macrophages which in turn become foam cells (Lucas and Greaves, 2001). OxLDL can also result in the increased expression of monocyte chemotactic protein-1 (MCP-1), and macrophage colony stimulating factor (MCSF), thereby promoting monocyte recruitment (Osterud and Bjorklid, 2003). Oxidized LDL can also uncouple eNOS and result in decreased NO bioavailability (Li and Mehta, 2005).

Additionally the long standing hyperglycemia and hyperinsulinemia along with the production of advanced glycation end products seen in diabetes, results in increased production of endothelin-1. Endothelin-1 not only increases vascular tone, but also activates the rennin-angiotensin system and promotes inflammation and smooth muscle proliferation (Creager *et al.*, 2003).

2.2.5.Oxidative stress and inflammation

C-reactive protein (CRP) is an acute phase protein elevated during systemic inflammation (Antoniades *et al.*, 2004). C-reactive protein is produced by the liver and its synthesis is stimulated by interleukin-6 (IL-6) secreted by macrophages (Whiteley *et al.*, 2009). Individuals with high CRP and IL-6 serum levels are at increased risk of developing CVD (May and Wang, 2007). The role of oxidative stress and inflammation in the progression of atherosclerosis cannot be over-emphasized. Atherosclerosis and its risk factors, including diabetes and hypercholesterolemia, are usually associated with low grade inflammation (Duncan *et al.*, 2003) which may in turn lead to the increased production of ROS within the vasculature (Libby, 2002; Packard and Libby, 2008). Free radicals can be cytotoxic and may cause damage to DNA, proteins, and lipids (Beckman and Koppenol, 1996). Despite the early observation that macrophages within atherosclerotic lesions give rise to foam cells containing cholesterol (Osterud and Bjorklid, 2003), the role of LDL in atherosclerosis was always questioned as LDL, by itself, did not appear to be atherogenic. Macrophages, reportedly, failed to internalize excess lipoprotein-cholesterol in vitro as LDL receptor is down regulated (Goldstein and Brown, 1977). It is now recognized that the oxidation of lipoprotein results in its conversion into a high uptake lipid form i.e. oxLDL (Vogiatzi *et al.*, 2009). This leads to endothelial dysfunction, smooth muscle de-differentiation and foam cell formation (Vogiatzi *et al.*, 2009). Peroxynitrite can also inactivate prostacyclin synthase, leading to decreased prostacyclin production and impaired vasodilatation (Zou *et al.*, 1999). A major source of super oxide anion is the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a complex enzyme with cytoplasmic and membrane components abundant in macrophages (Lassegue and Clempus, 2003). It has been found that many vascular cell types

express several components of the NADPH oxidase enzyme with oxidase activity, and may therefore contribute to oxidative injury (Yokoyama and Inoue, 2004).

2.3. Cigarette smoking

2.3.1.History of tobacco use

Tobacco leaves or *Nicotiana tabacum* were grown in Cuba and on several of the West Indies Isles, as well as the east coast of North America from Florida to Virginia for centuries. It was the Spanish and Portuguese merchants who first took tobacco plant seeds to Europe. In fact, the tobacco plant was named "Nicotina" after the French ambassador to Portugal Jean Nicot who introduced tobacco to the royal court of Paris in the 16^{th} century. Tobacco was used for the first time in England around the year 1565 and later was taken to Italy, Germany and Russia in the year 1600 (Hoffmann and Hoffmann, 1997).

2.3.2.Tobacco smoke streams and constituents

Smoking a cigarette releases an aerosol composed of a complex and dynamic mixture of compounds grouped into a vapor phase or a particulate phase (Hoffmann and Hoffmann, 1997). The vapor phase contains the volatile components such as carbon dioxide, carbon monoxide, acrolein, toluene, benzene and acetylene. The particulate phase contains more than 4000 compounds including more than 50 carcinogens (Hoffmann and Hoffmann, 1997; Smith *et al.*, 2003). These comprise the semi-volatile and non-volatile components of the aerosol such as nicotine, naphthalene and pyrene (Hoffmann and Hoffmann, 1997; Smith *et al.*, 2003). Three streams of cigarette smoke are generated as results of smoking i.e. main

stream smoke, side stream smoke and environmental tobacco smoke (ETS) (Borgerding and Klus, 2005). Main stream smoke is the smoke actively inhaled by the smoker from the filter end of a cigarette. Side stream smoke is generated from the burning end of a cigarette. Environmental tobacco smoke is a mixture of side stream smoke, smoke exhaled from the lung of a smoker and ambient air. It has been shown that the toxicity of aging sidestream smoke may be much greater than that of an equivalent mass dose of mainstream smoke (Schick and Glantz, 2006). In general, some compounds present in main stream smoke are similar to those found in ETS. However, differences in concentrations as well as physical and chemical properties have been reported. The exhaled portion of main stream smoke contributes only 15%-43% of the particulate matter of ETS with the rest originating from side stream smoke (Borgerding and Klus, 2005). Similarly, the majority of vapor phase constituents of ETS are derived from side stream smoke with only 1-13% coming from the exhaled portion of main stream smoke (Eatough DJ, 1990). The mean diameter of particles within main stream smoke is 0.27 +/- 0.03 µm, while the mean diameter of side stream smoke is 0.09 +/- 0.01 µm (Becquemin *et al.*, 2007). Approximately, 90-96% of the weight of the main stream smoke originates from the vapor phase, which contains 400-500 gaseous compounds (Hoffmann and Hoffmann, 1997; Hoffmann *et al.*, 2001). Oxygen, nitrogen and carbon dioxide are the main constituents of this phase (Hoffmann *et al.*, 2001). Several carcinogenic compounds including nitrogen oxides, isoprene, butadiene, benzene, styrene, formaldehyde, acetaldehyde, acrolein and furan can also be found (Hoffmann *et al.*, 2001). The remaining 5% of the smoke weight is present in the particulate phase (Hoffmann and Hoffmann, 1997; Hoffmann *et al.*, 2001). The particulate phase contains at least 3500

compounds. Nicotine is the major constituent of this phase comprising 0.2-0.6% of the total smoke weight (Hoffmann and Hoffmann, 1997; Hoffmann *et al.*, 2001).

The long term health effects of cigarette smoke constituents are largely controversial. For example, it has been shown that nicotine exposure not only decreases the production and bioavailability of NO (Fang *et al.*, 2003), but also increases the levels of asymmetric dimethyl arginine, an endogenous inhibitor of eNOS (Hamasaki *et al.*, 1997). However, others also found that nicotine promotes endothelial survival and release of vasodilators (Villablanca, 1998). Similarly, the exposure to carbon monoxide at low concentrations protects against oxidative stress and inhibits atherogenesis (Orozco *et al.*, 2007; Ryter *et al.*, 2006). Others also reported that carbon monoxide decreases oxygen delivery to tissues, blocks the mitochondrial respiratory chain (Kumar V, 2010), accelerates atherosclerosis (Paule *et al.*, 1976) and promotes cardiac hypertrophy (Sørhaug *et al.*, 2006). In addition the particulate phase contains many carcinogenic polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BAP) (Hoffmann and Hoffmann, 1997; Hoffmann *et al.*, 2001) which have deleterious health effects. These effects will be discussed later.

2.3.3.Tobacco smoke statistics and general health effects

Smoking continues to be a worldwide problem with negative health, economic and social effects. There are more than one billion smokers worldwide, with cigarette smoking killing more than 5 million people annually (Yach and Wipfli, 2006). Cigarette smoking is the leading cause of death in the US and accounts for nearly 1 of every 5 deaths each year (Mokdad *et al.*, 2004). In fact, the number of deaths related to tobacco smoking exceeds all

other deaths due to human immunodeficiency virus, illegal drug use, alcohol use, motor vehicle injuries, suicides, and murders combined (Center for Disease Control and Prevention, 2003; Mokdad *et al.*, 2004). In 1998, cigarette smoking killed 30,230 men and 17,351 women in Canada, of which 1,107 died due to second hand smoke exposure. The main causes of smoking-related deaths were lung cancer (13,951 deaths), ischemic heart disease (9,289 deaths) and chronic airways obstruction (6,457 deaths) (Makomaski Illing and Kaiserman, 2004).

The adverse effects of cigarette smoking involve almost every organ in the body. Chronic tobacco smoking is considered a major contributing factor towards many health problems, particularly cardiovascular and lung diseases (Center for Disease Control and prevention, 2003). Smoking increases the risk of many CVDs, including coronary heart disease, stroke, peripheral artery disease, hypertension, and aortic aneurysm (Bartal, 2001). A large epidemiologic study including 21 countries found that smokers under the age of 40 had a five times greater risk of heart attack compared to nonsmokers (Mahonen *et al.*, 2004). The risk of cerebral stroke is approximately doubled in smokers (Ockene and Miller, 1997).

The effects of cigarette smoking on the lungs have been thoroughly investigated. Smoking is a major risk factor for the development of chronic obstructive pulmonary disease (COPD) (Sharafkhaneh *et al.*, 2008). ETS exposure negatively influences the outcome of COPD in nonsmokers (Eisner *et al.*, 2006; Eisner *et al.*, 2009) Tobacco smoking is responsible for 90% and 80% of lung cancer deaths in women and men, respectively (Hecht, 1999). Countless number of epidemiological studies reported an association between tobacco smoking and several types of cancer (Sasco *et al.*, 2004). In fact, almost one third of cancer related deaths in the US are attributed to smoking (Shopland *et al.*, 1991). A positive

correlation has been shown between tobacco smoking and cancers of the lung, oral, cavity, pharynx, larynx, esophagus, pancreas, urinary bladder and renal pelvis (Sasco *et al.*, 2004). Tobacco smoking is also connected to sudden infant death syndrome and results in reduced memory and cognitive abilities in adolescent smokers (Anderson *et al.*, 2005; Jacobsen *et al.*, 2005). In comparison to smoking, the effects of ETS are less clearly established.

2.3.4.Smoking and cardiovascular disease

2.3.4.1.Epidemiologic evidence

Strong association between cigarette smoking and CVD has been reported in several studies. It is estimated that 30% of deaths due to coronary heart disease are related to cigarette smoking (Ambrose and Barua, 2004). Additionally, the risk of ischemic stroke is doubled in smokers compared to nonsmokers (Shinton and Beevers, 1989). Smokers are also at increased risk of claudication, abdominal aortic aneurysm and amputation than nonsmokers (Krupski, 1991). The exposure to ETS has also been linked to CVD. The risk of CVD is increased by 30% in people exposed to ETS (Center for Disease Control and Prevention, 2006).

2.3.4.2.Mechanisms of cigarette smoke induced cardiovascular disease

2.3.4.2.1.Endothelial dysfunction

Several studies reported the presence of morphological changes within endothelial cells after cigarette smoke or one of its constituents. Rat endothelial cells exposed *in vitro* to

nicotine at concentrations similar to those of smokers displayed subendothelial edema, swollen endoplasmic reticulum, and increased numbers of mitochondria (Pittilo *et al.*, 1990).

Aortic endothelial cells exposed *in vitro* to cigarette smoke extract displayed decreased eNOS activity and increased production of superoxide anion and peroxynitrite (Peluffo *et al.*, 2009a). Additionally, FMD was impaired in smokers compared to nonsmokers, an impairment which could be reversed by the administration of antioxidants for 165days (Peluffo *et al.*, 2009a). Similarly ETS exposure in 11 year–old children resulted in impaired endothelial function, measured as FMD, in a dose-dependent manner (Kallio *et al.*, 2007). The mechanism of FMD impairment and endothelial dysfunction is thought to be related to increased eNOS uncoupling, decreased NO production and bioavailability (Fang *et al.*, 2003).

2.3.4.2.2.Pulmonary inflammation and oxidative stress in smoking

The deposition of smoke particles within the lung is dependent on their particle size, with smaller particles penetrating deeper into the pulmonary system (Bernstein, 2004). Several *in vivo* and *in vitro* studies have shown that cigarette smoke exposure causes localized lung as well as systemic inflammation (Eileen, 2006). Cigarette smoke particles, first, cause injury to resident cells within the lung, stimulating them to release chemotactic factors, and inflammatory mediators. This results in the increased expression of adhesion molecules on the surface of endothelial cells as well as the activation of neutrophils to allow their entry into the lung.

Histologically, the lungs of smokers contain large numbers of pigmented and fluorescent macrophages associated with the ingestion of the tar component of cigarette smoke (Pauly *et al.*, 2005). Accumulations of polymorphonuclear cells have also been reported (Eileen, 2006). Tobacco smoking is also known to cause inflammation within the oral cavity (Taybos, 2003). The effects of cigarette smoke exposure on markers of oxidative stress have also been evaluated in exhaled air and bronchoalveolar lavage (BAL) fluid from smokers. In breath condensate from smokers the levels of 8-isoprostane, a marker of lipid peroxidation, as well as the levels of hydrogen peroxide are increased (Guatura *et al.*, 2000; Montuschi *et al.*, 2000). Within BAL fluid the levels of oxidized glutathione and superoxide are also increased (Morrison *et al.*, 1999; Nagai *et al.*, 2006). The breath condensate of smokers also has increased levels of leukotriene B4 (LTB-4) and IL-6 (Carpagnano *et al.*, 2003). High levels of IL-8 have also been reported (Tanino *et al.*, 2002). Within the peripheral blood the level of LTB-4 increased significantly after acute exposure to cigarette smoke (Kobayashi *et al.*, 1988). IL-8 and LTB-4 attract neutrophils toward the alveoli (Kumar V, 2010). In animal models, the exposure to cigarette smoke results in the recruitment of neutrophils and macrophages toward the lung (Kilburn and McKenzie, 1975). In lung tissue, the expression levels of macrophage inflammatory protein-1 (MIP-1), and macrophage chemoattractant protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) have been shown to increase after cigarette smoke exposure (Wright *et al.*, 2002). MCP-1 and MIP-1 are chemotactic for macrophages. The release of TNF-α from alveolar macrophages is increased when they are exposed to a high concentration of cigarette smoke extract (Churg *et al.*, 2003). Additionally, peripheral blood mononuclear cells and macrophages exposed *in vitro* to cigarette smoke release interleukin-1β (IL-1β) and TNF-α (Ryder *et al.*, 2002). IL-1β and

TNF-α can enhance the recruitment of inflammatory cells by increasing the expression, synthesis, and release of several cytokines and cell adhesion molecules by other cells (Dinarello, 2000; Mills *et al.,* 1999). Epithelial cells also contribute to cigarette smoke-induced lung inflammation. Moreover, normal human bronchial epithelial cells exposed to cigarette smoke condensate display increased mRNA levels of IL-1β, IL-8 and GM-CSF (Hellermann *et al.,* 2002). This is thought to be mediated by the activation of extracellular signal-regulated protein kinase-1/2 and nuclear factor κB (Hellermann *et al.,* 2002). Cigarette smoke exposure increases airway permeability by affecting the integrity of tight junctions between epithelial cells (Morrison *et al.,* 1999). The disruption of tight junctions results in the accumulation of smoke constituents within the subepithelial region (Bhalla *et al.,* 2009). The slow clearance of smoke particles within this region causes not only increased retention of carcinogens within the lung, but also extended release of particulates from the lung toward the systemic circulation. This slow and continuous release of smoke constituent particles causes a short cigarette smoke exposure to mimic a chronic exposure scenario (Bhalla, 1999). Additionally, it has been shown that oxidants in the gas phase of cigarette smoke have the ability to pass through alveolar wall and raise systemic oxidative stress (Yamaguchi *et al.,* 2007). The increased systemic inflammatory response and oxidative stress due to active or passive smoking alters endothelial function and increase the risk for CVD.

Cigarette smoke exposure is an important risk factor for COPD (Higgins, 1991). Smokers with COPD often have histological evidence of chronic bronchitis and alveolar emphysema (Selman, 2003). The inflammatory response to cigarette smoke exposure includes the release of elastases and metalloproteinases from macrophages and neutrophils within the lung (Churg *et al.,* 2008). These include neutrophil elastase, metalloproteinase-9 (MMP-9),

produced by macrophages and neutrophils, and metalloproteinase-12 (MMP-12) produced by macrophages (Churg *et al.*, 2008). It has been shown that guinea pigs given an inhibitor of MMP-9 and MMP-12 are protected from cigarette smoke-induced alveolar emphysema (Churg *et al.*, 2007). These enzymes not only destroy the extracellular matrix but also help regulate inflammation through the release of cytokines and chemokines (Van Lint and Libert, 2007). In comparison to smoking the effects of ETS are less clearly established.

2.4. Polycyclic aromatic hydrocarbons (PAHs)

The polycyclic aromatic hydrocarbons are a group of lipophilic and persistent environmental contaminants that arise as by-products of combustion of organic matter (Tithof *et al.*, 2002). This family of contaminants contains compounds like naphthalene and anthracene (Haritash and Kaushik, 2009). Polycyclic aromatic hydrocarbons are used extensively in laboratory animals to induce neoplastic transformation in tissues and the prototypical carcinogen is a reactive metabolite of the PAH, BAP (Nebert *et al.*, 2000)

2.4.1.Mechanism of action of polycyclic aromatic hydrocarbons

Many of the actions of PAHs are mediated by a ligand-activated transcription factor known as the aryl hydrocarbon receptor (Ahr) (Korashy and El-Kadi, 2006). Ahr binds to the xenobiotic response element on DNA, and causes transcriptional induction of dioxin-responsive genes such as the detoxification enzyme cytochrome P450 1A1 enzyme (CYP1A1) (Korashy and El-Kadi, 2006). The first step in the metabolism of PAHs is epoxidation, which is mediated by CYP1A1 (Levin *et al.*, 1982). PAH epoxides are then

converted to diols through the enzyme epoxide hydrolase. The oxidation of PAHs to epoxides results in the formation of highly reactive metabolites which can form protein and DNA adducts leading to cancer development (Levin *et al.*, 1982). Furthermore, quinone metabolites of PAHs are capable of redox cycling and can generate large amounts of ROS (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). The expression of CYP1A1 in tissues is also used as a marker of exposure to contaminants that activate Ahr. Ahr stimulation within the lung, liver or endothelial cells results in high expression levels of CYP1A1 (Brunnberg *et al.*, 2006; Kopf and Walker, 2010). This can be used in mechanistic investigations of toxicity since only the parent PAH compounds can activate Ahr, while their metabolites generally cannot (Nebert *et al.*, 2000).

2.4.2.Polycyclic aromatic hydrocarbons within cigarette smoke

The particulate phase of cigarette smoke contains many of the PAHs like benzo[a]antharacene, benzo[b]fluoranthene, benzo[j]fluoranthene and BAP (Hoffmann and Hoffmann, 1997). The levels of PAHs within side stream smoke are reported to be 10 times higher than that in main stream smoke (Lodovici *et al.*, 2004), potentially making PAH-mediated effects more relevant to ETS than to smoking.

2.4.3.Polycyclic aromatic hydrocarbons and cardiovascular disease

There is accumulating evidence that PAH exposure enhances the development of atherosclerosis. It has been shown that in the absence of toxicant, an increase in shear stress results in CYP1A1 induction and cell cycle arrest of endothelial cells which is mediated by

Ahr activation (Han *et al.*, 2008). However, there is virtually no knowledge of the normal physiological role, if any, of Ahr in CV physiology. In human beings, a correlation between the exposure to PAHs and the development of ischemic heart disease has also been found (Burstyn *et al.*, 2005). High levels of DNA adducts associated with PAHs have been found in atherosclerotic plaques of human beings (Izzotti *et al.*, 1995). Moreover, patients with advanced atherosclerotic lesions have higher levels of aortic DNA adducts compared to patients with mild or moderate atherosclerotic lesions (Binková *et al.*, 2002). Experimentally, the exposure to PAHs accelerates the development of atherosclerosis (Penn and Snyder, 1988), which could be related to increased oxidative stress (Nebert *et al.*, 2000), and increased adhesiveness of endothelial cells (Oesterling *et al.*, 2008). The most widely studied member of the PAH family of compounds is BAP. BAP was also the first carcinogen identified in cigarette smoke (Commins *et al.*, 1954). Similar to other PAHs, BAP is metabolized to 7,8-epoxide by the enzyme CYP1A1. The enzyme epoxide hydrolase converts 7,8-epoxide to 7,8-diol. It is then the secondary oxidation of 7,8-diol by CYP1A1 which converts BAP to the ultimate carcinogen BAP 7,8-diol-9,10-epoxide (Moorthy *et al.*, 2003). Additionally, BAP can be metabolized to quinone structures by CYP1A1 which can undergo redox cycling to produce ROS (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). The administration of BAP to apolipoprotein E- knockout mice results in endothelial proinflammatory effects i.e. induces expression of MCP-1 (Knaapen *et al.*, 2007), and accelerated lipid peroxidation (Godschalk *et al.*, 2003). Inhaled PAHs also cause inflammatory reactions within the lung (Belitskaya-Levy *et al.*, 2007; Pei *et al.*, 2002). Therefore, it seems plausible that PAHs such as BAP may be responsible for the increased CVD risk posed by smoking tobacco and chronic exposure to ETS.

2.5. Resveratrol (RES)

Natural antioxidants, within fruits and vegetables, such as RES, sulphoraphane and many others, have received increasing attention in recent years because of their role in protecting against CVD, cancer, and inflammation (Kode *et al.*, 2008). Resveratrol is a polyphenolic compound found in several types of fruits with antioxidant (Kawada *et al.*, 1998), anti-inflammatory (Donnelly *et al.*, 2004), and anti-carcinogenic properties (ElAttar and Virji, 1999). The interest in RES arose after a study in the early 1990s which pointed to a link between the low mortality among the French population from coronary heart disease compared to North Americans, despite much higher smoking rates, and the consumption of red wine; the so-called "French paradox" (Renaud and de Lorgeril, 1992). The cardio-protective property of red wine was attributed to RES. Additionally, it has been reported that RES attenuates cigarette smoke-induced oxidative stress and proinflammatory phenotypic alterations within endothelial cells (Csiszar *et al.*, 2008). Our interest in RES, versus other natural antioxidants, stems from the claim that red wine consumption, and thus RES, is protective for the cardiovascular system and may also reverse the negative effects of cigarette smoke.

2.5.1.Resveratrol structure and metabolism

Resveratrol or 3,5,4'-trihydroxystilbene is a natural antioxidant present in grapes, peanuts and different species of berries such as blueberries, bilberries, and cranberries (Burns *et al.*, 2002). The skin of grapes contains 50-100 µg/g RES (Ector *et al.*, 1996). Despite its high absorption (70%), only trace amounts of unchanged RES is found in plasma, which is

due to extensive and rapid liver metabolism. A dose of 25mg (110 µmol) given orally resulted in a peak concentration of resveratrol and metabolites in plasma of 491±90 ng/ml. However, unchanged RES levels were very low in plasma (< 5ng/ml). The half-life of unchanged RES was 8-14 minutes while the half-life for its metabolites was 9.2±0.6h. Three metabolic pathway of RES have been identified i.e. sulfation, glucuronic acid conjugation and hydrogenation (Walle *et al.*, 2004). The biologic effects of these metabolites are unknown.

2.5.2.Resveratrol's health effects and mechanisms of action

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases (Lin *et al.*, 2000). The member silent information regulator 2 (Sir2) is found in budding yeast *Saccharomyces cerevisiae.* Calorie restriction to this yeast has been shown to prolong its lifespan, which is thought to be mediated by Sir2 activity (Lin *et al.*, 2000). Sirt-1 is mammalian homologue of Sir2 (Cohen *et al.*, 2004). Sirt-1 deacetylates histones associated with several important transcriptional factors and thus alters their function. For example Sirt-1 deacetylates the protein P-53 making it inactive, preventing cells from undergoing apoptosis and therefore increasing their life span (Yang *et al.*, 2006). Resveratrol is known to be a potent activator of Sirt-1 and hence is claimed to increase life span in mammals (Borra *et al.*, 2005). Resveratrol is reported to be antiatherosclerotic with much of this effect attributed to its antioxidant activity (Sarfaraz and Rohit, 2009). Resveratrol can decrease plasma lipid concentration in apolipoprotein E-deficient, hypercholesterolemic rats (Fukao *et al.*, 2004). Through copper chelation, RES also decreased the copper-mediated oxidation of

LDL thus inhibiting atherosclerosis (Belguendouz *et al.*, 1997). Additionally RES decreases vascular oxidative stress by inhibiting vascular NADPH oxidase enzyme (Orallo *et al.*, 2002). The recruitment of monocytes to the vasculature may also be reduced by RES treatment. Human umbilical vein endothelial cells incubated with RES and later with bacterial lipopolysaccharide display not only decreased oxidative stress, but also reduced VCAM-1 mRNA and protein expression (Carluccio *et al.*, 2003). Resveratrol also decreases the angiotensin II-induced proliferation of vascular smooth muscle cells (Haider *et al.*, 2003). It has been reported that RES is also a vasodilator since phenylephrine-induced aortic contraction is inhibited by RES treatment (Chen and Pace-Asciak, 1996). Moreover, RES has been found to antagonize the effects of the potent vasoconstrictor, endothelin (Yang *et al.*, 1998). Resveratrol is also known to be an Ahr antagonist as it blocks the induction of CYP1A1 by dioxins (Ciolino *et al.*, 1998).

A few studies are doubtful about the cardioprotective effects of RES. It has been reported that RES given to rats does not prevent peroxidation of serum lipids and has no effect on lipoprotein profile (Turrens *et al.*, 1997). Others have also found that RES possesses pro-oxidant properties and in the presence of transition metals, such as copper, can result in oxidative DNA break down (de la Lastra and Villegas, 2007). Two pharmaceutical companies i.e. Amgen and Pfizer, recently challenged the claims of beneficial health effects of RES reporting that RES is not an activator of Sirt-1 (Beher *et al.*, 2009; Pacholec *et al.*, 2010) and does not decrease serum glucose levels in mice fed high fat diet (Beher *et al.*, 2009; Pacholec *et al.*, 2010). Therefore, not only is the mechanism of action of RES in the CV system unclear, but also the health benefits are yet to be clearly established.

3. Hypotheses and research objectives

3.1. Hypothesis

1. Pigs are a good model to study and detect cardiovascular and lung changes after chronic ETS exposure.
2. Chronic ETS exposure results in systemic inflammation and oxidative stress leading to vascular endothelial dysfunction and cardiac dysfunction as well as lung inflammation and emphysema in swine.
3. Chronic exposure to BaP, a PAH within cigarette smoke, will mimic the ETS-induced health effects in swine.
4. Resveratrol, a natural antioxidant, will protect against the ETS- induced health effects in swine.

3.2. Objectives

3.2.1 To examine the feasibility of using pigs as a model to detect cardiovascular and lung changes after chronic ETS exposure.

3.2.2 To examine the effects of chronic ETS exposure on markers of systemic oxidative stress and inflammation, vascular endothelial function, cardiac function, lung inflammation and emphysema.

3.2.3 To examine the health effects of chronic exposure to BAP, a PAH found in ETS, and to check if it mimics the effects induced by ETS.

3.2.4 To examine whether RES, a natural antioxidant, protects against the ETS induced health effects.

4. Pulmonary inflammation and Cytochrome P450 1A1 activity are linked to endothelial dysfunction after ETS exposure, but not after benzo-a-pyrene injection in juvenile pigs

4.1. Introduction

The adverse health effects of cigarette smoke involve almost every organ system in smokers (Alberg, 2008; Bartal, 2001). However, cardiovascular and respiratory effects are most prevalent including diseases such as atherosclerosis, coronary artery disease, asthma, and COPD (Armani *et al.*, 2009; Bhalla *et al.*, 2009). Exposure to second-hand tobacco smoke, also referred to as ETS, poses an increased risk for many of these same diseases. In fact, smoking and ETS exposure are considered the number one preventable risk factor contributing to CVD.

Endothelial cells play a key role in the regulation of vascular tone and impairment of normal endothelial function is thought to be an early step in the development of all CVD, including in smokers (Luscher, 1994). Endothelial dysfunction causes impaired vascular relaxation which in turn increases peripheral vascular resistance and contributes to atherosclerosis (Walsh *et al.*, 2009). An increase in vascular tone and hemodynamic overload would also cause myocardial systolic dysfunction because the heart has to work harder to eject blood against the increased afterload (Borlaug *et al.*, 2009). Furthermore, a prolonged increase in afterload causes left ventricular hypertrophy and alteration in left ventricular extracellular matrix (Verma and Solomon, 2009) which would then contribute to diastolic dysfunction. In support of this, several studies have provided evidence that acute and chronic exposure to cigarette smoke impair left ventricular diastolic function (Alam *et al.*, 2002;

Gulel *et al.*, 2007). Therefore, assessing endothelial function has become increasingly important as a prognostic clinical tool and method used in mechanistic investigations of cardiovascular pathophysiology (Harris *et al.*, 2010). Flow mediated dilation is an endothelium dependent ultrasound technique which measures the ability of conduit arteries to relax and expand in response to a shear stress stimulus. Flow mediated dilation has been used as an early predictor for CVD (Martin, 2009) and is thought to be mediated largely by NO. Flow mediated dilation has been shown to be impaired in smokers which might be related to decreased NO bioavailability (Celermajer *et al.*, 1996; Karatzi *et al.*, 2007; Peluffo *et al.*, 2009b). However, the effect of ETS on endothelial function is less clearly established.

Cigarette smoke-induced oxidative stress is a major pathway contributing to the development of CVD (Minicucci *et al.*, 2009; Vardavas and Panagiotakos, 2009). Peroxynitrite is produced as a result of the interaction between superoxide anion and NO (Ischiropoulos and al-Mehdi, 1995; van der Vliet *et al.*, 1995) which leads to reduced NO bioavailability and impaired vasodilation (Peluffo *et al.*, 2009b). Cigarette smoke exposure also results in increased serum 3-nitrotyrosine levels, which is generated by the nitration of tyrosine residues within proteins by peroxynitrite (Kunitomo *et al.*, 2009). Additionally, epidemiologic studies have shown that cigarette smokers have higher levels of CRP in their serum, compared to non smokers (Antoniades *et al.*, 2004; Hanyu *et al.*, 2009). C-reactive protein is an acute phase protein elevated during inflammation (Antoniades *et al.*, 2004) and individuals with high CRP serum levels are at increased risk of developing CVDs (May and Wang, 2007).

Cigarette smoke is a mixture of more than 4000 compounds and contains different classes of toxic chemicals including PAHs (Borgerding and Klus, 2005; Ding *et al.*, 2008). Although

nicotine is the constituent that is responsible for cigarette smoke addiction and is known to exert many stimulatory actions on the cardiovascular system (Rahman and Laher, 2007), it does not explain the chronic effects of cigarette smoke or ETS exposure (Rahman and Laher, 2007). Polycyclic aromatic hydrocarbons are lipophilic compounds which result from the combustion of organic material (Tithof *et al.*, 2002). Many of the actions of PAHs are mediated by a ligand activated transcription factor known as the Ahr (Korashy and El-Kadi, 2006). By binding to the xenobiotic response element on DNA, this transcription factor causes transcriptional induction of dioxin-responsive genes such as the detoxification enzyme cytochrome P450 1A1 enzyme (CYP1A1) (Korashy and El-Kadi, 2006). The metabolism of PAHs, such as BAP, by CYP1A1 results in the formation of highly reactive metabolites which, in turn, can form adducts with DNA and protein (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). Additionally, PAH metabolites can be metabolized to quinone structures which can undergo redox cycling to produce ROS (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). Tobacco smoke also contains carbon monoxide and many unstable, reactive molecules that would increase the levels of carboxyhemoglobin (CO-Hb) and methemoglobin (MetHb), respectively, after tobacco smoke exposure (Borland *et al.*, 1985). In addition to known vasodilatory effects of CO (Achouh *et al.*, 2008), it has been shown that endothelial cells produce peroxynitrite after CO exposure (Thom *et al.*, 1997), suggesting that CO by itself can also stimulate oxidative stress. Therefore, there are multiple chemical sources of oxidative stress arising from cigarette smoke.

Oxidative stress can also be produced as a result of inflammation (van der Vaart *et al.*, 2004). Active cigarette smoking is known to induce lung inflammation as evidenced by the presence of neutrophils and macrophages within alveoli (van der Vaart *et al.*, 2004).

Inflammation within the airways is also known to promote decreased lung function, asthma and respiratory irritation in children and adults passively exposed to ETS (Chan-Yeung and Dimich-Ward, 2003; Salvi and Barnes, 2009). Pulmonary neutrophils and macrophages not only generate ROS and inflammatory cytokines, but also release extracellular matrix degrading enzymes *i.e*. elastases which contribute to lung emphysema (Sharafkhaneh *et al.*, 2008). Although the exact identity and mechanisms responsible are not clear, these inflammatory mediators originating in the lung may also travel systemically to cause further inflammation in distal tissues, particularly in the endothelium of the vasculature (Tamagawa *et al.*, 2008)**.** Thus, pulmonary inflammation caused by ETS exposure may be a central mechanism responsible for the increased incidence of both pulmonary disease as well as CVD.

We hypothesized that chronic exposure to ETS would result in lung and systemic inflammation which in turn would result in increased oxidative stress, endothelial dysfunction, impaired FMD, increased blood pressure, impaired left ventricular function and increased broncho-alveolar elastase associated with emphysema. We also hypothesized that these effects could be mimicked by BAP exposure. In order to evaluate this hypothesis we first randomized 8 castrated male pigs into two groups and performed daily 1-hr ETS- or sham-exposures for 28 days. Another 8 pigs received either a daily intravenous BAP or vehicle injection for 7 days. Flow mediated dilation, left ventricular end systolic volume (ESV), end diastolic volume (EDV), ejection fraction (EF), mean arterial pressure, systolic pressure, diastolic pressure, CO-Hb, oxyhemoglobin (OxyHb), MetHb, total hemoglobin (TotalHb), serum nitrate/nitrite (NOx), nitrotyrosine, CRP, liver CYP1A1 and lung CYP1A1 were measured. Total and differential cell count, and neutrophil elastase level in

bronchoalveolar lavage (BAL) fluid were performed at the termination of both experiments. Histological and morphometric analyses were also performed on the abdominal aorta, brachial, and coronary arteries, while lung, liver and heart were evaluated for histolopathology.

4.2. Materials and methods

4.2.1.Animals

All protocols were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Council at the University of Saskatchewan. In each experiment, 2 week old, castrated male landrace pigs (10-12 kg) were obtained from Prairie Swine Center Inc (Saskatoon, SK) and randomized into exposure or sham groups (4/group). The pigs were group-housed with each treatment group kept in separate pens under a 12 hr dark/12 hr light cycle. Pigs were fed normal pig starter chow (Federated Co-Operatives Ltd, Saskatoon, Canada) and water *ad libitum* except during exposure, injection or cardiovascular assessment.

4.2.2.Environmental tobacco smoke (ETS) and benzo[a]pyrene (BAP) exposure

In the first experiment, a single cigarette manual smoking machine from CH Technologies Inc (Westwood, NJ, USA) was used to generate ETS (mainstream plus side stream smoke). The machine was adjusted to a rate of 3 puffs/minute with 57 ml/puff and a 2 second duration. The ETS, mixed with unfiltered indoor air, was then pumped into a 500 gallon polyethylene plastic water tank modified to include inflow and out flow ports and a sealed, removable plexiglass door in which unsedated, unrestrained pigs (n=4 per group) were exposed. Pumps controlling inflow were set at 6 L/min. A total of 12 cigarettes (Filtered Canadian Classics, Rothmans, Benson & Hedges, Canada) were burned over the span of 1 hr every day for 28 days. Sham-exposed pigs (n=4) were placed in the same cleaned chamber for an hour but with an unlit cigarette attached to the smoking machine. The

total particulate count in the chamber was assessed for the ETS-exposed and sham-exposed pigs using a SKC constant airflow pump (Universal 224-PCXR, Eighty Four, PA) fitted with pre-weighed mixed cellulose ester filter (0.08 μm, SKC Inc., Eighty Four, PA). Total particulates were sampled continuously for one hour at 2L/min. Carbon monoxide levels were also monitored in both groups using a T40 Rattler CO monitor (Industrial scientific, Corp, Oakdale, USA) placed inside the chamber for the duration of the exposure. The air levels of O_2/CO_2 within the chamber were measured using a Criticare Poet IQ multiparameter gas monitor (Criticare Systems, Inc., Waukesha, USA). In the second experiment, pigs received a daily intravenous injection of BAP (5 mg/kg) (Sigma-Aldrich, St. Louis, USA, 96%) via the anterior vena cava for 7 days. Control pigs received the same volume of a vehicle control (0.2 ml/kg tricaprylin, T9126, Sigma-Aldrich, St. Louis, USA, 99%) daily for the same period.

4.2.3.Flow mediated dilation (FMD), echocardiography, blood pressure, and blood gas evaluation

Because FMD has not previously been reported in pigs, FMD measurements were validated (n=8 pigs) in preliminary experiments. Pigs were first sedated by receiving an intramuscular injection of azaperone (2.2 mg/kg, Stresnil™ Merial Inc, Canada). Pigs were placed in left lateral recumbency. The left brachial artery was visualized using a SonoSite 180 Plus ultrasound unit with a 5.0 MHz linear array transducer (SonoSite Canada Inc., Markham, Ont., Canada). The probe was placed on the medial aspect of the distal third of the left radius, approximately 10 centimeters from the axilla. The blood pressure cuff was applied directly distal to the ultrasound area. The brachial artery was visualized at baseline

(unoccluded) in order to extract a minimum of 3 images that were averaged and this average value was used as the baseline diameter. The blood pressure cuff was then inflated to ~30 mmHg above systolic pressure for 4 minutes. Following cuff release the brachial artery was visualized for 150s. All ultrasound views were recorded using a digital video camera for the duration of the post-occlusion time and uploaded to a computer. Single digital images of the brachial artery at diastole were extracted at 15s, 30s, 45s, 60s, 75s, 90s, 105s, and 120s after cuff release using Adobe Premiere Elements (Adobe Inc., San Jose, CA, USA). The perimeter (P) of the brachial artery at each time point was traced and measured using Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA) (Figure 4.1) and converted to a diameter using the formula: diameter = P/π . All measurements of digital ultrasound images were performed blinded. The flow mediated dilation was calculated using the formula:

$$\% \text{ FMD} = \frac{(\text{post-occlusion diameter}) - (\text{baseline diameter})}{(\text{baseline diameter})} \times 100\%$$

For exposure experiments, at the beginning of each ultrasound session, pigs were sedated as previously described and the rectal temperature was measured using a thermometer immediately after ETS /sham exposure or BAP/vehicle injection. The flow mediated dilation evaluation was performed as described above. The left ventricular end-systolic volume (ESV) and end-diastolic volume (EDV) were measured using an HP SONOS 100CF ultrasound machine. A 5.0 MHz cardiac transducer was used in the right parasternal long-axis view to visualize the left ventricular outflow tract during systole and diastole. For each end point two measurements were taken and then averaged. The ejection fraction (EF) was calculated using the formula:

$$EF = [(EDV - ESV) / (EDV)] \times 100\%$$

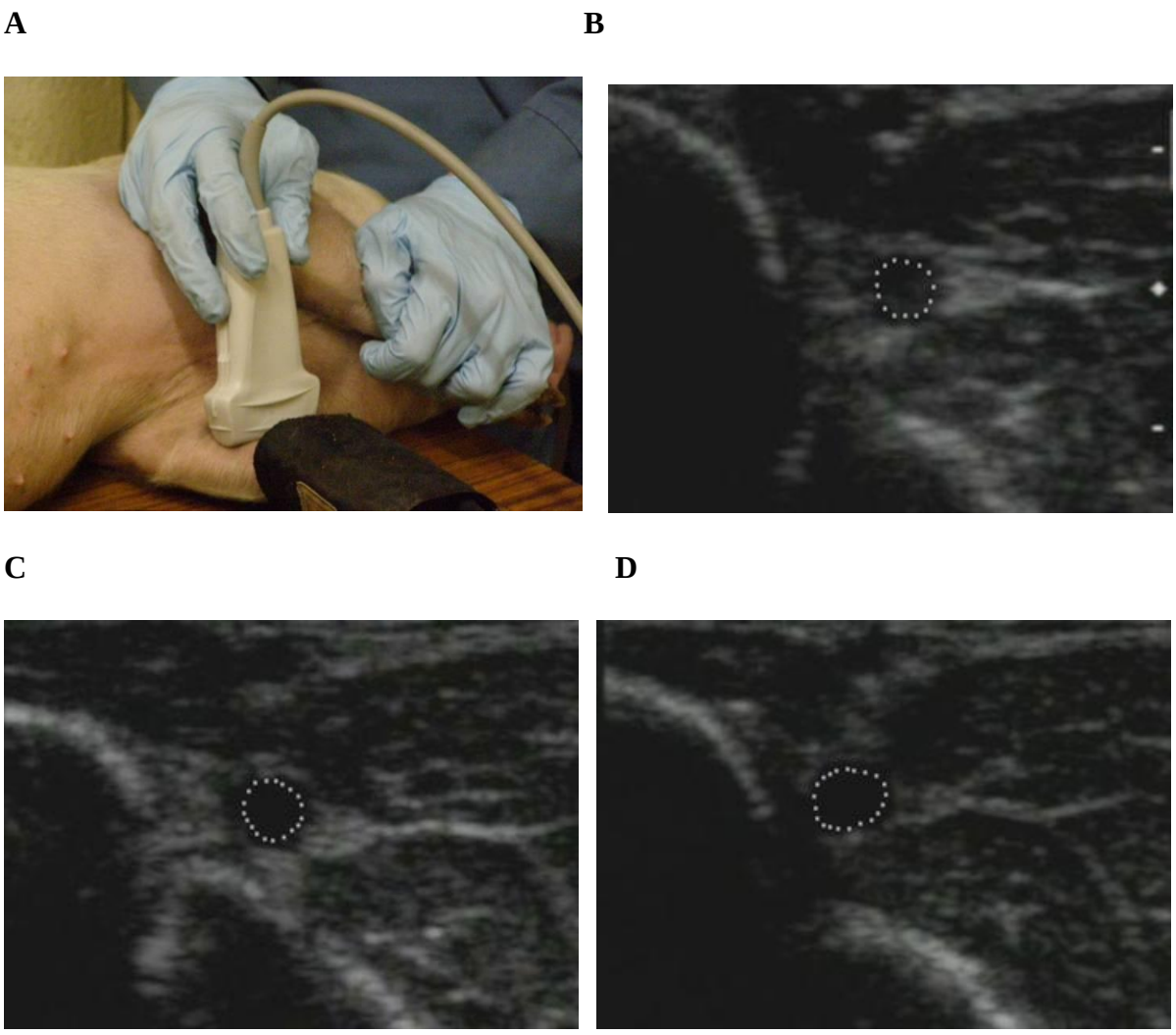


Figure 4.1: Ultrasound probe and cuff placement for brachial artery flow mediated dilation evaluation (A). Brachial artery ultrasound images at baseline (B), 60s (C) and 90s (D) after cuff release.

After vascular and cardiac ultrasound, a Memo Diagnostic High Definition Oscillometer (S + B medVET, Markham, Ont. Canada) was placed on the distal end of the right hind limb to measure systolic pressure, diastolic pressure, mean arterial pressure (MAP) and heart rate. The mean of at least 3 consecutive blood pressure readings from each pig was used from a given session for statistical analyses. Finally, blood gases were measured in venous blood collected from the jugular vein into blood gas syringes (Radiometer PICO 50; Radiometer; Copenhagen, Denmark). Oxygenated hemoglobin (oxyHb), carboxy-hemoglobin (CO-Hb), methemoglobin (MetHb) and total hemoglobin (TotalHb) levels were measured using a Rapidlab 865 blood gas analyzer (Bayer Diagnostics, East Walpole, MA, USA). Ultrasound, blood pressure and blood gas measurements were taken at baseline (*i.e*. Day 0 or prior to starting the exposures), day 1, day 7, day 14, day 21 and day 28 for the ETS experiment. In the second experiment the same parameters were measured at baseline, day 1 and day 7.

4.2.4.Plasma nitrate/nitrite, cotinine, nitrotyrosine, C-reactive protein (CRP), and ethoxyresorufin-o-deethylase (EROD) activity

Blood samples were collected from the right anterior vena cava from all pigs at baseline and on the last day of experiments into EDTA or serum Vacutainers for plasma and serum, respectively, and stored on ice until spun at 4000xg for 10 minutes. Plasma and serum were aliquoted, then stored at -80°C until use in assays. Plasma nitrate/nitrite (NOx) levels were assessed using a commercially available enzyme-based kit (NO Quantitation Kit, Active Motif North America, Carlsbad, USA). Plasma cotinine (Bio-Quant, Inc, San Diego, USA), plasma nitrotyrosine (Cell Sciences, Canton, USA) and serum CRP (CRP; Geneway Biotech,

CA, USA) levels were measured using commercially available enzyme-linked immunosorbent assays. Liver and lung homogenates from all pigs were analyzed for EROD activity, as previously described (Weber *et al.*, 2002).

4.2.5.Bronchoalveolar lavage (BAL) fluid differential, total cell count and leukocyte elastase activity

At the end of each experiment pigs were euthanized by an intravenous injection with pentobarbital sodium (Euthanol; 100 mg/kg). Bronchoalveolar lavage was performed by infusing 40 ml of normal saline into the isolated trachea and lungs, with 20 ml of the lavage fluid retrieved in this process. First a hemocytometer was used to determine the total white blood cell count, then a cytospin preparation stained with Wright's solution was used to determine the differential cell count in each BAL sample. Bronchoalveolar lavage fluid leukocyte elastase activity was measured using a synthetic substrate *i.e*. N-succinyl-$(ala)_3$-*p*-nitroanilide (Sigma-Aldrich, ON, Canada) as previously described (Castillo *et al.*, 1979).The activity of the enzyme was measured in units in which one unit of leukocyte elastase released one nanomole of *p*-nitrophenol per sec from BOC-L-alanine *p*-nitrophenyl ester at pH 6.5 at 37 °C.

4.2.6.Histological and morphometric analyses

Following euthanasia, tissues from the heart, lung, liver as well as aorta, coronary and brachial arteries were collected and placed in 10% formalin. Tissues were paraffin-embedded, serial 5 μm sections cut, stained with hematoxylin and eosin (H& E), and

mounted with Micromount (Surgipath, AB, Canada). All tissues were evaluated for histopathological changes by a veterinary pathologist. For each artery, morphometric digital analyses were performed on 3 images captured from 3 different sections. Histological micrographs were taken using an Olympus BX41 microscope, attached to an Olympus DP71 digital camera, and captured using DP controller software (Olympus Canada Inc., Markham, Canada). Image Pro Plus7 was used to trace and measure the internal perimeter and wall thickness (WT) of each of the 3 images, and averages were then determined. From each perimeter the luminal diameter (LD) was calculated using the formula: diameter = P/π , and the ratio of LD to WT (LD/WT) was also determined.

4.2.7.Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA, USA) or Systat 11 (Chicago, IL, USA). In order to correct for individual variation in baseline measurements that occurred among pigs, all subsequent measurements were normalized by dividing by their corresponding baseline values from the same pig. Differences among groups were analyzed by two-way analysis of variance with treatment and time as factors followed by Bonferroni posteriori tests. Terminal end points were analyzed by an unpaired t-test. A p-value < 0.05 was considered significant. All data were expressed as mean ± standard error of the mean (SEM).

4.3. Results

4.3.1.Flow mediated dilation (FMD) validation

Because FMD has not previously been reported in pigs, the time course of brachial artery dilation in 8 pigs was visualized before and after occlusion for four minutes. The diameter of the brachial artery increased gradually after cuff release until reaching a maximum at 90s post-occlusion (Figure 4.2). Based on this preliminary experiment, the 90s time-point was used for all subsequent FMD evaluations. Flow mediated dilation at this 90s time point was 18.8 ± 1.0 % in normal, castrated male pigs.

4.3.2.Flow mediated dilation (FMD), echocardiography, blood pressure, and blood gases evaluation

Chamber conditions during ETS and sham exposures are summarized in Table 4.1. In general, the levels of CO and total particulates were higher during ETS exposures than sham exposures. Chamber O_2 levels decreased after ETS exposure compared to sham exposure, but remained >21%. In contrast, CO_2 levels remained below the detection limit (<0.5%) during both exposures. Serum cotinine levels after 4-weeks exposure were significantly higher in ETS-exposed compared to sham-exposed pigs (Table 4.1).

In order to assess the effects of ETS on cardiovascular function *in vivo*, ultrasound and blood pressure assessments were performed weekly in pigs. A significant decrease in FMD was noted over time in both ETS and sham exposed pigs ($p<0.05$ for time factor in 2-way ANOVA; Figure 4.3). However, FMD was further decreased by ETS-exposure ($p<0.05$ for treatment factor), becoming significantly different from sham-exposed pigs in posteriori tests by Day 7 and continuing for the remainder of the experiment (Figure 4.3).

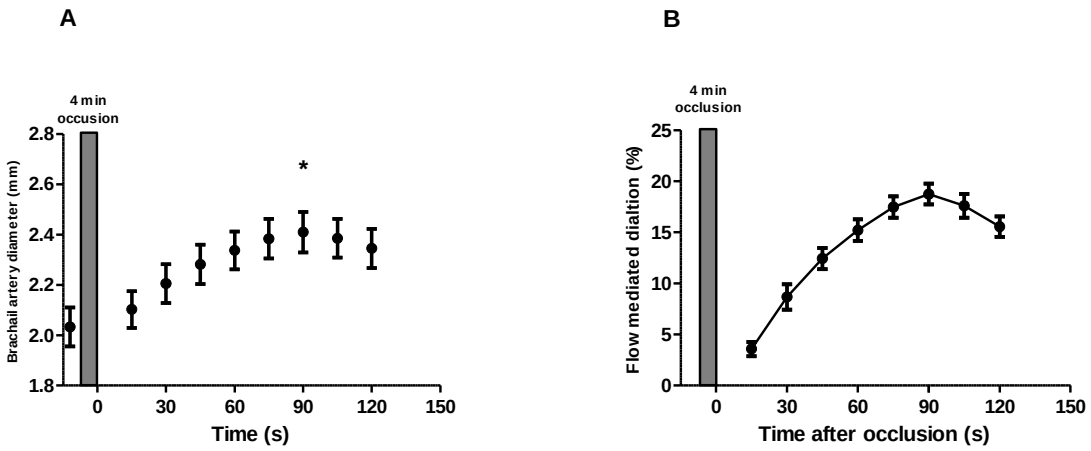


Figure 4.2: A. Brachial artery diameter at baseline and 15, 30, 45, 60, 75, 90, 105 and 120s post cuff release in normal, castrated male pigs (n=8). **B.** Flow mediated dilation (FMD) measured at baseline and at 15, 30, 45, 60, 75, 90, 105 and 120s post cuff release (n=8). Data are expressed as mean ± standard error of the mean. *$p<0.05$ compared to pre-occlusion diameter in Bonferroni posteriori test after one-way analysis of variance. From these data 90s post cuff release was chosen for FMD evaluation because it was the time at which maximum relaxation was seen.

Table 4.1: Environmental tobacco smoke (ETS) exposure conditions and terminal plasma cotinine concentrations. Conditions in the exposure chamber were determined in preliminary experiments. Temperature and carbon monoxide (CO) were continuously monitored for 1 hr during ETS or sham exposure, and were expressed as a mean of 3 readings taken every 20 minutes. Particulates were sampled continuously during 1 hr sham or ETS exposure and are expressed as total particulate matter/hr. A total of 11-12 cigarettes were burned during the daily ETS exposure. Blood samples, collected after exposure, on day 28 were used for plasma cotinine quantitation using an enzyme-linked immunosorbent assay. Data are expressed as mean ± standard error. *$p<0.05$ determined using an unpaired t-test with Welch's correction.

	Sham	**ETS**
Temperature	24ºC	23ºC
CO (mean)	0	136 ppm
Particulates	30 μg/m^3	110 μg/m^3
O_2	24%	21%
CO_2	<0.5%	<0.5%
Plasma Cotinine	2.1 ± 0.4 ng/ml	51.6 ± 4.8 ng/ml*

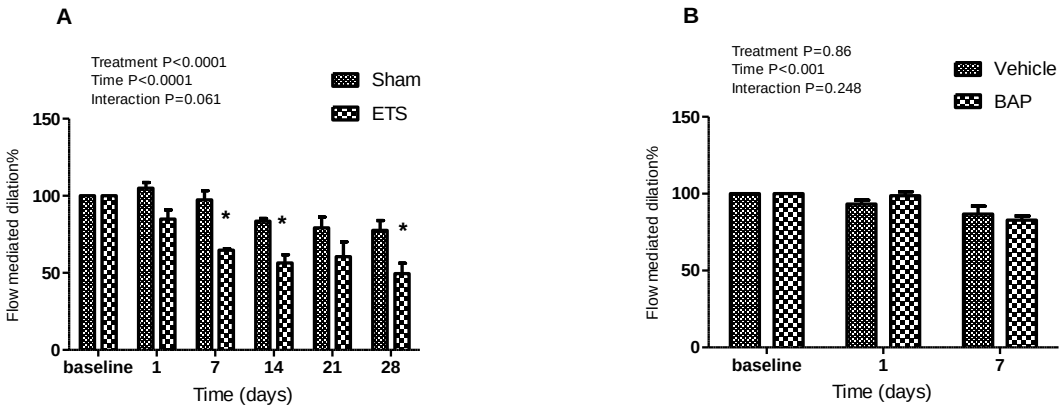
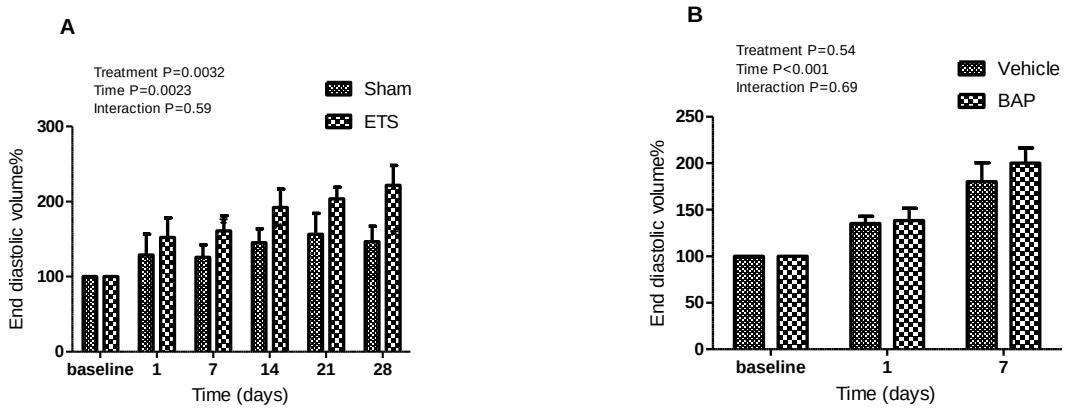


Figure 4.3: A. Changes in flow mediated dilation (FMD), normalized to pre-exposure values, over time after daily 1-hr environmental tobacco smoke (ETS) exposure or sham exposure for 28 day or **B.** after daily benzo[a]pyrene (BAP) or vehicle injection for 7 days. Data for each animal (n=4 per group for both experiments) are normalized to the baseline (or pre-exposure) response in the same individual. Data were analyzed by two way analysis of variance (ANOVA) with treatment and time as factors, and expressed as means ± standard errors of the means (SEM). *$p<0.05$ compared to sham-exposure by Bonferroni posteriori test after two-way ANOVA.

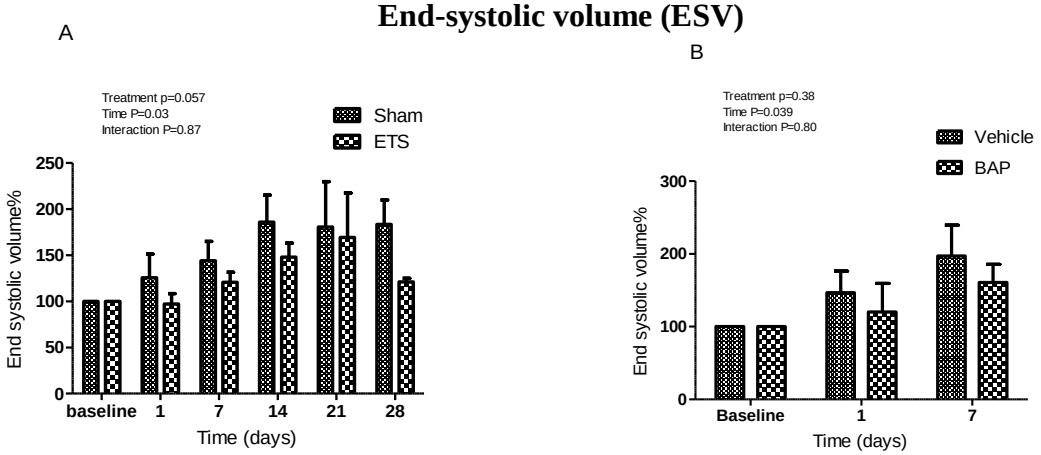
Similar to that observed in the ETS exposure, FMD significantly decreased over time as the pigs grew ($p<0.05$ for time factor; Figure 4.3). However, in contrast to ETS, BAP exposure did not significantly impair FMD compared to vehicle injection at any time (Figure 4.3).

Left ventricular end-diastolic volume (EDV) and end-systolic volume (ESV), but not ejection fraction (EF) increased in a time-dependent manner as the pigs grew in both experiments (Figure 4.4, Table 4.2 and Table 4.3). More importantly, a significant overall increase in left ventricular EDV was found after ETS exposure compared to sham exposure ($p<0.05$ for treatment factor in 2-way ANOVA), but modified Bonferroni posteriori tests failed to detect any significant pair-wise effects (Figure 4.4). A trend pointing to a decrease in left ventricular ESV and an increase in EF was found after ETS exposure compared to sham exposure (Figures 4.4). In contrast, BAP injection had no significant effect on left ventricular EDV, ESV, or EF compared to vehicle injection (Figures 4.4). Heart and body weight were determined only at the termination of both experiments. No significant difference was found in heart weight, body weight or heart/body weight ratio between ETS exposed and sham exposed pigs, or BAP injected and vehicle injected pigs (Table 4.4). Blood pressure (systolic, diastolic, mean and pulse pressures) was not significantly altered over time or between ETS and sham exposed pigs (Table 4.5). Similarly no significant difference was found in blood pressure after BAP or vehicle injection (Table 4.6). Venous hemoglobin levels were altered by both ETS and BAP exposure, but in different manners (Figure 4.5, Table 4.7 and Table 4.8). Specifically, OxyHb tended to increase over time in sham-exposed pigs (Figure 4.5), but not in ETS-exposed pigs.

End-diastolic volume (EDV)



End-systolic volume (ESV)



Ejection fraction (EF)

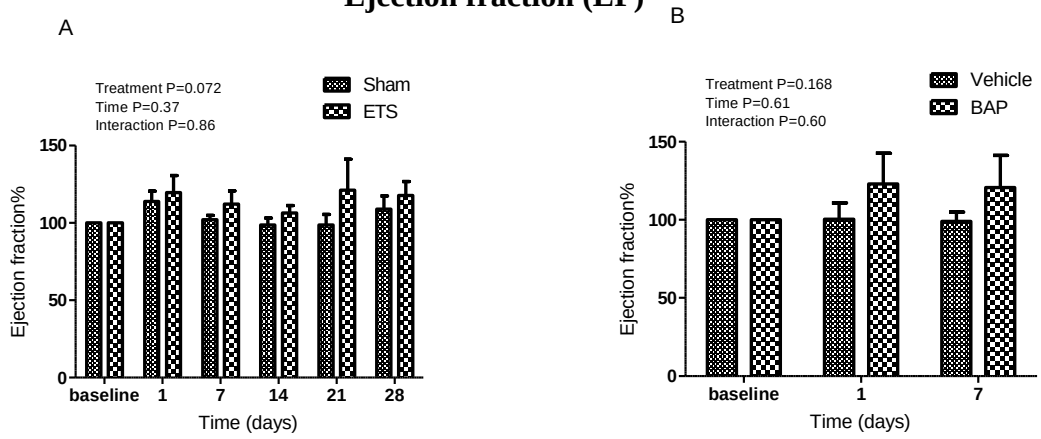


Figure 4.4: Changes in left ventricular end diastolic volume (EDV; top panels), end-systolic volume (ESV; middle panels) and ejection fraction (EF; bottom panels) after daily 1-hr environmental tobacco smoke (ETS) or sham exposure for 28 days (A) or after daily benzo[a]pyrene (BAP) or vehicle injection for 7 days (B). Data for each animal (n=4 per group in both experiments) are normalized to the baseline (or pre-exposure) response in each individual and expressed as mean ± standard error of the mean. Effects of time and treatment were analyzed using two-way analysis of variance followed by modified Bonferroni posteriori tests, as appropriate.

Table 4.2: Absolute values obtained for end diastolic volume (EDV), end systolic volume (ESV), and ejection fraction (EF) determined over time after daily environmental tobacco smoke (ETS) or sham exposure for 28 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were all performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	ESV (ml)		**EDV (ml)**		**EF%**	
	Sham	**ETS**	**Sham**	**ETS**	**Sham**	**ETS**
Baseline	3.1 ± 0.3	4.7 ± 0.5	7.4 ± 0.4	12.0 ± 1.6	60 ± 2	57 ± 6
Day 1	3.7 ± 0.5	4.4 ± 1.3	11.1 ± 1.2	14.4 ± 1.9	68 ± 3	66 ± 1
Day 7	4.4 ± 0.5	5.5 ± 0.2	11.8 ± 1.2	14.3 ± 0.4	61 ± 2	62 ± 3
Day 14	5.6 ± 0.6	6.8 ± 0.5	14.2 ± 2.0	16.5 ± 0.4	59 ± 2	59 ± 3
Day 21	5.2 ± 1.0	7.4 ± 1.3	15.0 ± 0.9	17.8 ± 1.9	59 ± 34	66 ± 5
Day 28	5.5 ± 0.5	5.7 ± 0.6	16.3 ± 1.7	16.6 ± 0.6	65 ± 4	66 ± 2

Table 4.3: Absolute values obtained for end diastolic volume (EDV), end systolic volume (ESV), and ejection fraction (EF) determined over time after daily injection with benzo[a]pyrene (BAP) or vehicle for 7days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were all performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	ESV (ml)		**EDV (ml)**		**EF (%)**	
	Vehicle	**BAP**	**Vehicle**	**BAP**	**Vehicle**	**BAP**
Baseline	1.9 ± 0.3	2.0 ± 0.2	4.4 ± 0.4	4.4 ± 0.2	60 ± 4	54 ± 5
Day 1	2.5 ± 0.2	2.2 ± 0.5	5.9 ± 0.2	6.0 ± 0.4	58 ± 4	64 ± 6
Day 7	3.3 ± 0.2	3.1 ± 0.4	7.8 ± 0.2	8.7 ± 0.6	60 ± 2	63 ± 6

Table 4.4: Heart weight, body weight and heart / body weight ratio after daily environmental tobacco smoke (ETS) or sham exposure for 28 days (n=4 pigs/group), or benzo[a]pyrene (BAP) or vehicle injection for 7 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. No significant differences were detected between ETS and sham-exposed pigs as well as no differences between BAP- and vehicle-injected pigs using t-tests.

	Heart weight (Kg)	**Body weight (Kg)**	**Heart / Body weight (%)**
Sham	0.209 ± 0.04	31.75 ± 6.3	0. 65 ± 0.01
ETS	0.205 ± 0.01	35.0 ± 0.41	0. 59 ± 0. 03
Vehicle	0.071 ± 0.002	12.94 ± 0.51	0. 55 ± 0. 03
BAP	0.077± 0.003	12.83 ± 0.36	0. 61 ± 0. 04

Table 4.5: Absolute values for systolic, diastolic, mean arterial and pulse pressure (mmHg) over time after daily environmental tobacco smoke (ETS) or sham exposure for 28 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were all performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	Systolic pressure (mm Hg)		**Diastolic pressure (mm Hg)**		**Mean arterial pressure (mm Hg)**		**Pulse pressure (mm Hg)**	
	Sham	**ETS**	**Sham**	**ETS**	**Sham**	**ETS**	**Sham**	**ETS**
Baseline	109 ± 6	124 ± 20	49 ± 4	55 ± 6	70 ± 4	79 ± 11	60 ± 7	69 ± 14
Day 1	99 ± 5	116 ± 11	51 ± 2	55 ± 5	69 ± 3	77 ± 7	48 ± 4	61 ± 6
Day 7	117 ± 11	147 ± 14	54 ± 4	59 ± 6	76 ± 3	90 ± 8	63 ± 7	88 ± 10
Day 14	117 ± 13	123 ± 16	52 ± 8	43 ± 3	75 ± 10	71 ± 7	64 ± 6	80 ± 15
Day 21	134 ± 16	118 ± 13	54 ± 4	59 ± 12	82 ± 7	80 ± 12	80 ± 15	59 ± 5
Day 28	122 ± 13	136 ± 9	53 ± 5	63 ± 3	78 ± 5	89 ± 4	69 ± 14	73 ± 9

Table 4.6: Systolic, diastolic, mean arterial, and pulse pressure, over time after daily benzo[a]pyrene (BAP) or vehicle injection for 7 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were all performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	Systolic pressure		**Diastolic pressure**		**Mean arterial pressure**		**Pulse pressure**	
	Vehicle	**BAP**	**Vehicle**	**BAP**	**Vehicle**	**BAP**	**Vehicle**	**BAP**
Baseline	139 ± 19	115 ± 8	72 ± 13	57 ± 1	96 ± 15	78 ± 3	67 ± 7	58 ± 9
Day 1	107 ± 11	111 ± 10	61 ± 7	56 ± 2	78 ± 8	76 ± 5	47 ± 6	56 ± 7
Day 7	135 ± 11	111 ± 3	64 ± 4	47 ± 2	89 ± 6	69 ± 2	72 ± 8	64 ± 2

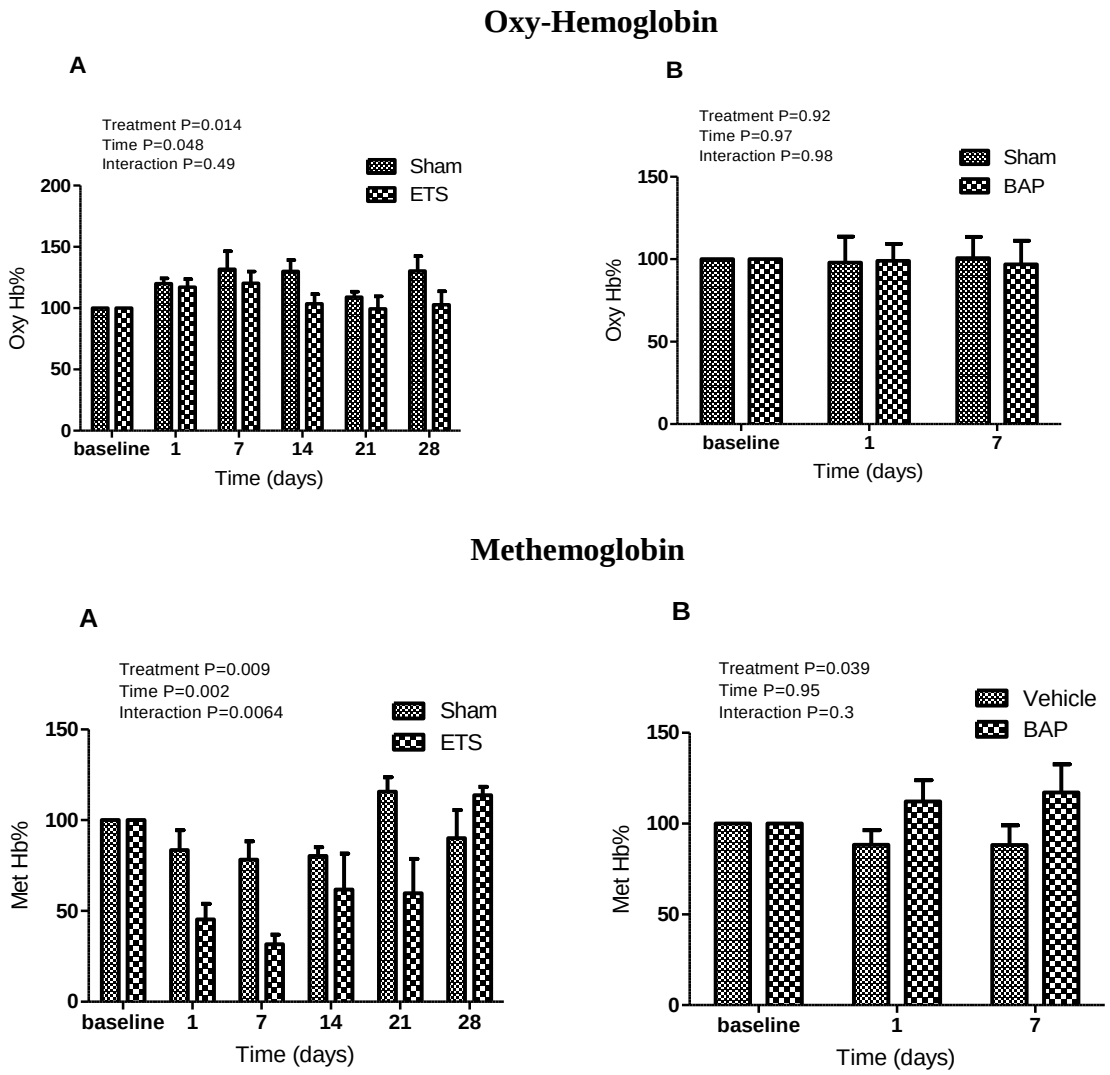


Figure 4.5: Changes in venous oxyhemoglobin (OxyHb; top panels) and methemoglobin (MetHb; bottom panels) after daily 1-hr environmental tobacco smoke (ETS) or sham exposure for 28 days (A) or after daily benzo[a]pyrene (BAP) or vehicle injection for 7 days (B). Data for each animal (n=4 per group in both experiments) are normalized to the baseline (or pre-exposure) response in the same individual and expressed as mean ± standard error of the mean. Effects of time and treatment were examined using two-way analysis of variance followed by Bonferroni posteriori tests.

Table 4.7: Oxyhemoglobin (OxyHb) carboxyhemoglobin (CO-Hb), methemoglobin (MetHb) and total hemoglobin (TotalHb) in venous blood over time after daily environmental tobacco smoke (ETS) or sham exposure for 28 days (n=4 pigs/group). Data are expressed as means ± standard errors of the means. Absolute values are shown for qualitative evaluation only since statistical analyses were performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	OxyHb (%)		**CO-Hb (%)**		**MetHb (%)**		**TotalHb (mg/L)**	
	Sham	**ETS**	**Sham**	**ETS**	**Sham**	**ETS**	**Sham**	**ETS**
Baseline	65.7 ± 3.1	72.7 ± 4.7	0.10 ± 0.00	0.10 ± 0.00	2.4 ± 0.1	2.4 ± 0.1	113 ± 4	102 ± 7
Day 1	79.2 ± 6.6	85.0 ± 6.4	0.15 ± 0.05	0.65 ± 0.41	2.0 ± 0.3	1.1 ± 0.2	108 ± 2	95 ± 4
Day 7	85.4 ± 7.0	87.3 ± 7.9	0.20 ± 0.04	0.45 ± 0.26	1.8 ± 0.2	0.8 ± 0.1	108 ± 1	101 ± 1
Day 14	85.2 ± 6.5	75.1 ± 7.4	0.15 ± 0.03	0.23 ± 0.05	1.9 ± 0.1	1.5 ± 0.5	108 ± 5	106 ± 5
Day 21	71.4 ± 2.8	72.8 ± 9.2	0.05 ± 0.03	0.13 ± 0.06	2.7 ± 0.2	1.4 ± 0.4	109 ± 4	103 ± 3
Day 28	85.2 ± 7.6	73.9 ± 7.5	0.10 ± 0.04	0.05 ± 0.03	2.1 ± 0.4	2.7 ± 0.1	118 ± 7	106 ± 8

Table 4.8: Oxyhemoglobin (OxyHb) carboxyhemoglobin (CO-Hb), methemoglobin (MetHb) and total hemoglobin (TotalHb) in venous blood over time after daily benzo[a]pyrene (BAP) or vehicle injection for 7 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	OxyHb (%)		**CO-Hb (%)**		**MetHb (%)**		**TotalHb (mg/L)**	
	Vehicle	**BAP**	**Vehicle**	**BAP**	**Vehicle**	**BAP**	**Vehicle**	**BAP**
Baseline	86.6 ± 5.3	75.0 ± 6.5	0.10 ± 0.00	0.10 ± 0.00	2.3 ± 0.2	2.5 ± 0.1	97 ± 10	120 ± 8
Day 1	82.8 ± 10.4	73.0 ± 3.4	0.15 ± 0.03	0.10 ± 0.00	2.0 ± 0.1	2.8 ± 0.3	97 ± 10	84 ± 21
Day 7	85.2 ± 5.4	71.4 ± 7.7	0.18 ± 0.05	0.15 ± 0.03	2.0 ± 0.2	2.9 ± 0.3	71 ± 27	66 ± 15

This led to an overall decreased level of venous OxyHb in ETS-exposed compared to sham-exposed pigs ($p<0.05$ for treatment factor in 2-way ANOVA), but posteriori tests failed to detect specific time points where differences were significant (Figure 4.5). In contrast, BAP injection had no significant effect on venous OxyHb levels at any time (Figure 4.5; Table 5.8). Venous MetHb was significantly decreased in the earlier time-points of ETS exposure compared to sham-exposure ($p<0.05$ for both time and treatment factors in 2-way ANOVA), but then returned to pre-exposure levels by day 28 of this experiment (Figure 4.5). This biphasic response of MetHb in the ETS-exposed pigs created a significant interaction between the time and treatment variables (Figure 4.5). In contrast, BAP injection caused a significant increase in MetHb levels compared to vehicle-injected pigs without any interaction between the factors in ANOVA analyses (Figure 4.5). Carboxyhemoglobin and TotalHb were not significantly altered by ETS exposure (Table 4.7) or BAP injection (Tables 4.8) compared to their respective controls.

4.3.3.Plasma nitrate/nitrite, nitrotyrosine, C-reactive protein (CRP), and ethoxyresorufin-o-deethylase (EROD) activity

Overall, serum nitrate/nitrite levels (an indicator of NO production) were highly variable and no significant changes were observed after either ETS or BAP exposure compared to their corresponding control groups (Table 4.9). However, BAP injection caused a significant increase in nitrotyrosine (an indicator of NO inactivation by superoxide free radical via peroxynitrite formation) compared the vehicle-injected group by day 7 (Figure 4.6). An increase of similar magnitude in nitrotyrosine was also observed at the end of 28 days of ETS exposure compared to sham exposure, but was not quite statistically significant (Figure 4.6).

C-reactive protein (an acute phase inflammatory protein) was significantly elevated after both ETS and BAP exposure compared to their respective controls (Figures 4.7). Inhalation exposure to ETS caused a significant increase in lung, but not liver EROD activity compared to sham-exposed pigs (Figure 4.8). In contrast, intravenous BAP injection caused the opposite result, with a significant increase in liver, but not lung EROD activity compared to vehicle injection (Figure 4.8).

4.3.4.Bronchoalveolar lavage (BAL) fluid total white blood cell count, BAL differential cell count, BAL leukocyte elastase activity and tissue histology

The total WBC count in BAL fluid was significantly increased after ETS (Figure 4.9), but not after BAP exposure compared to their respective controls (Figure 4.9). Macrophages were the only inflammatory cell type found within BAL fluid from both ETS and sham-exposed pigs. A subjective evaluation of lung sections showed an increase in intraalveolar macrophages in ETS-exposed pigs compared to sham-exposed pigs (Figure. 4.10). No significant difference was found in BAL fluid leukocyte elastase concentration after either ETS or BAP exposure compared to their respective controls (Table 4.10). No histopathological evidence of lung emphysema was found in any of the samples from these experiments (Figure 4.10). Furthermore, no histopathological lesions were detected in any other tissue that was evaluated; liver, heart or arteries (Figure 4.11). Finally, there was no significant difference in arterial wall thickness or arterial diameter (evaluated as luminal diameter, wall thickness or a ratio of the two) in aorta, brachial artery or coronary artery after ETS or BAP exposure compared to their respective control tissues (Table 4.11).

Table 4.9: Total serum nitrate/nitrite levels over time after daily environmental tobacco smoke (ETS) or sham exposure for 28 days, and benzo[a]pyrene or vehicle injection for 7days. Data are expressed as mean ± standard error of the mean and n=4 pigs/group for both experiments. ND: Not determined. No statistically significant differences were detected using separate two-way analyses of variances for each experiment (time and treatment as factors).

	Total nitrate/nitrite (µM)			
	Sham	**ETS**	**Vehicle**	**BAP**
Baseline	4.9 ± 2.6	4.6 ± 0.7	7.7 ± 0.8	9.5 ± 2.8
Day 1	5.2 ± 1.2	4.3 ± 1.6	5.7 ± 1.7	7.9 ± 1.9
Day 7	ND	ND	1.7 ± 0.6	5.4 ± 3.4
Day28	1.4 ± 0.7	0.9 ± 0.2	------	------

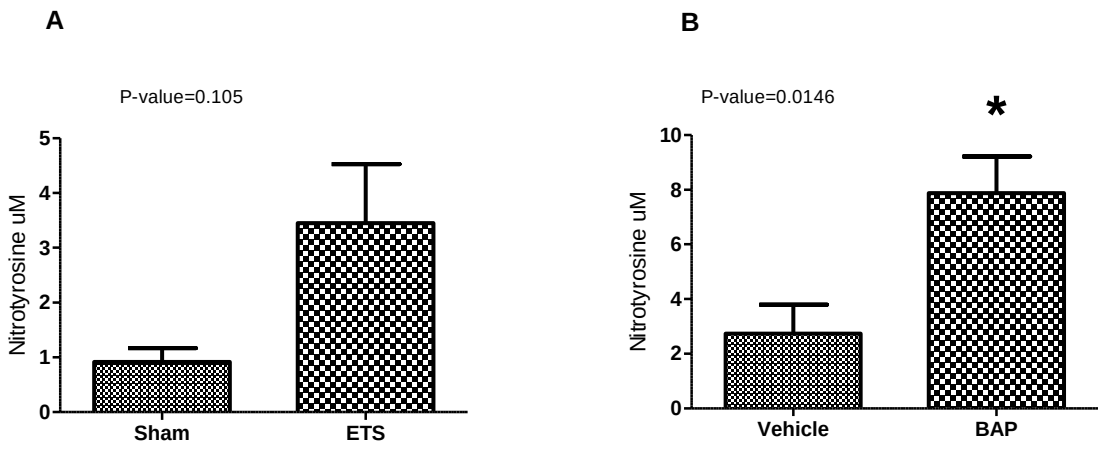


Figure 4.6: A. Serum nitrotyrosine levels after 28 days of environmental tobacco smoke (ETS) or sham exposure or **B.** after 7 days of benzo[a]pyrene (BAP) or vehicle injection. Data are expressed as mean ± standard error of the mean and n=4/group in both experiments. *$p < 0.05$ in unpaired t-test with Welch's correction.

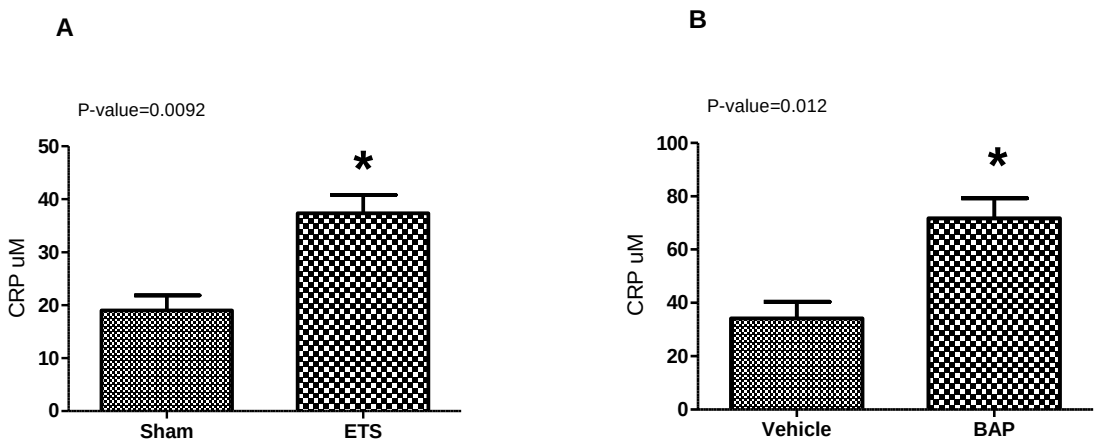


Figure 4.7: A. Serum C-reactive protein levels after 28 days of environmental tobacco smoke (ETS) or sham exposure or **B.** after 7 days of benzo[a]pyrene (BAP) or vehicle injection. Data are expressed as mean ± standard error of the mean and n=4/group in both experiments. *$p < 0.05$ in unpaired t-test with Welch's correction.

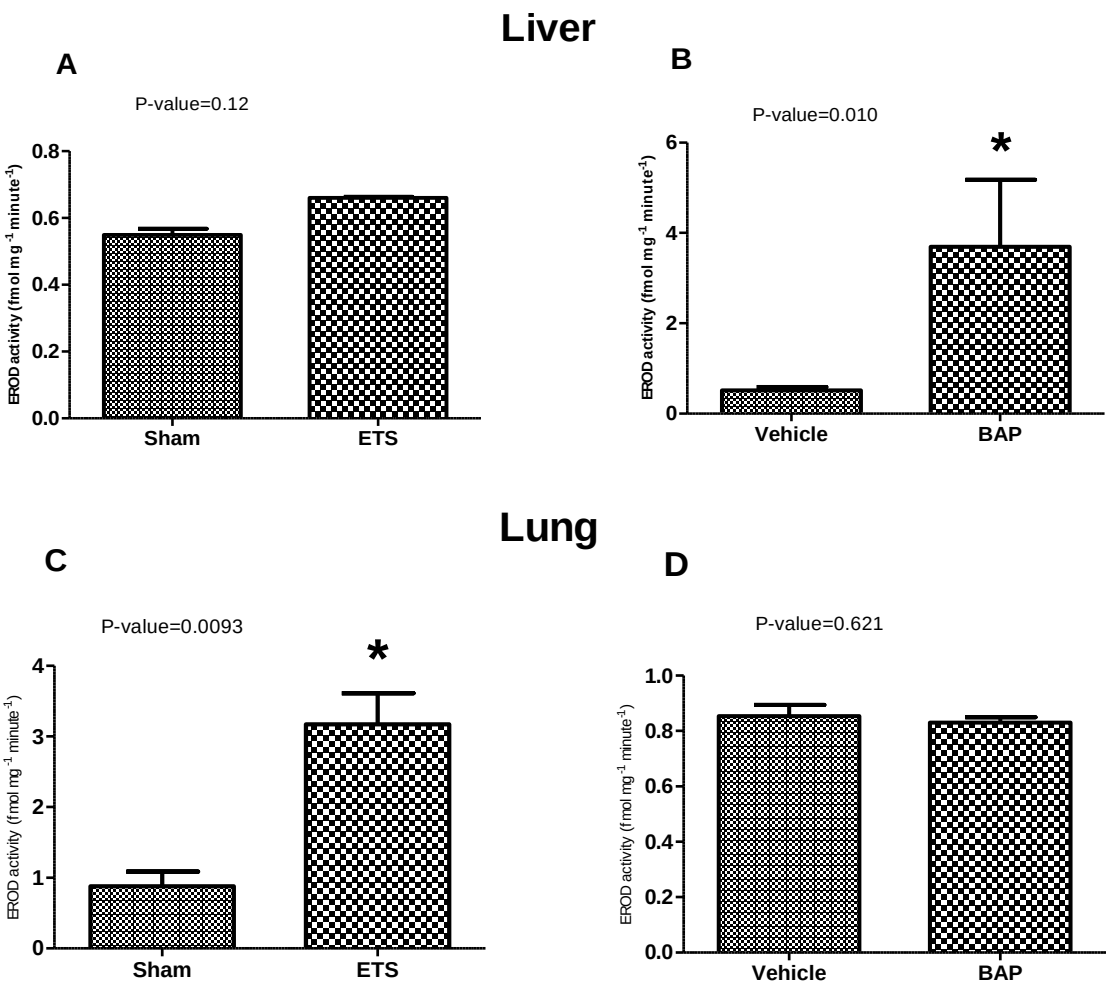


Figure 4.8: Ethoxyresorufin-o-deethylase (EROD) activity in liver (A,B) and lung (C,D) microsomes prepared from pigs after 28 days of environmental tobacco smoke (ETS) or sham exposure (A,C) as well as after 7 days of benzo[a]pyrene (BAP) or vehicle injection (B,D). Data are expressed as mean ± standard error of the mean and n=4 pigs/group for both experiments. *$p < 0.05$ in unpaired t-test with Welch's correction.

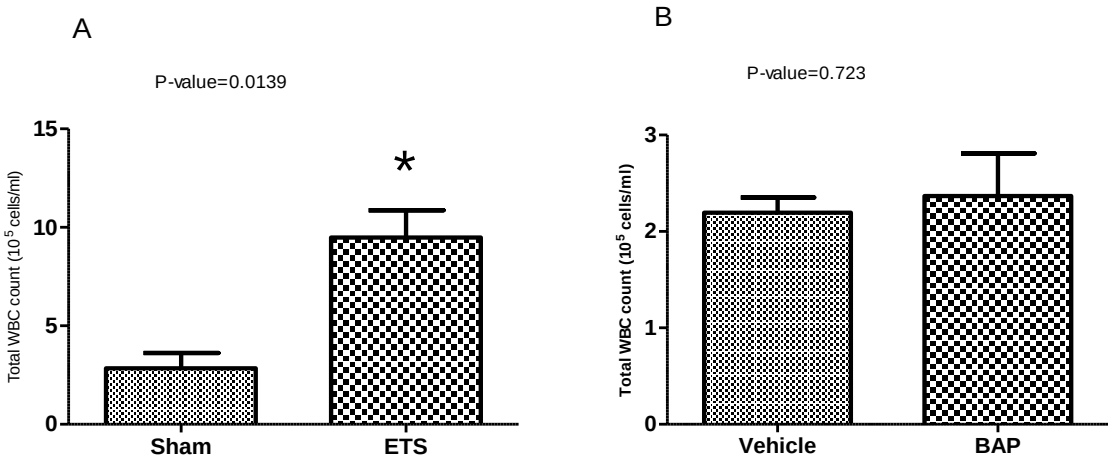


Figure 4.9: A. Total white blood cell (WBC) count in bronchoalveolar lavage (BAL) fluid collected from pigs after 28 days of environmental tobacco smoke (ETS) or sham exposure or **B.** after 7 days of benzo[a]pyrene (BAP) or vehicle injection. Data are expressed as mean ± standard error of the mean and n=4 pigs/group in both experiments. *$p < 0.05$ in unpaired t-test with Welch's correction.

Table 4.10: Leukocyte elastase concentration in bronchoalveolar lavage (BAL) fluid collected from pigs after 28 days of environmental tobacco smoke (ETS) or sham exposure, or after benzo[a]pyrene (BAP) or vehicle injection for 7days. Data are analyzed by an unpaired t test with Welch's correction and expressed as mean ± standard error of the mean. U: standard Unit of elastase activity.

Treatment type	Leukocyte elastase (U)	p-value
Sham	0.36 ± 0.06	0.1350
ETS	0.65 ± 0.16	
Vehicle	0.29± 0.05	0.1962
BAP	0.43 ± 0.08	

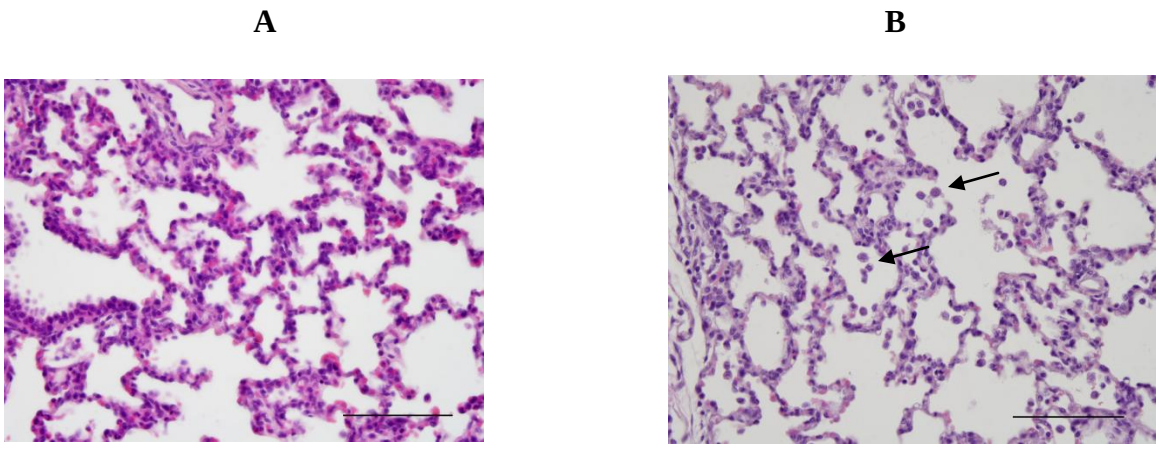
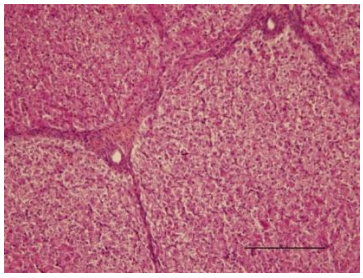
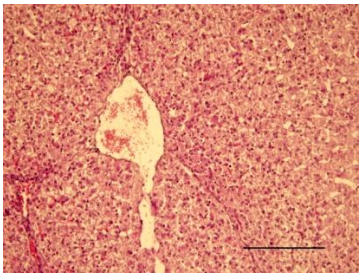


Figure 4.10: Representative histological micrographs of hematoxylin/eosin-stained lung tissue collected from pigs after 28 days of sham (A) or environmental tobacco smoke (ETS) (B) or exposure. Qualitative histological assessment of alveoli revealed an increase in intraalveolar macrophages after ETS exposure (arrows). Bar = 100 µm.

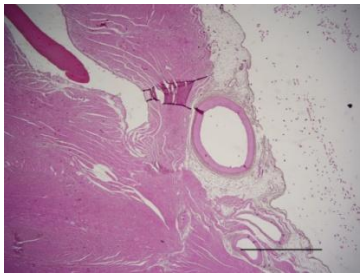
A



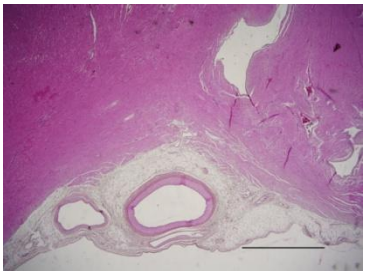
B



C

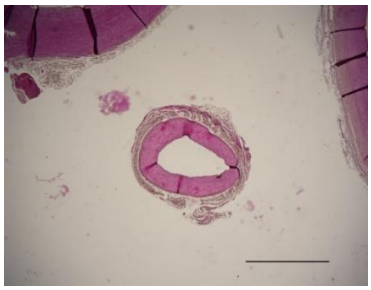


D

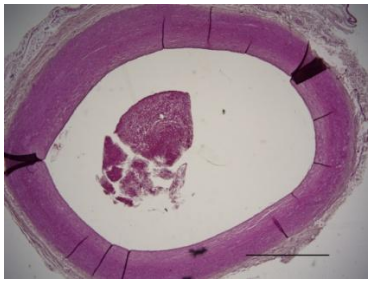


E

F



G



H

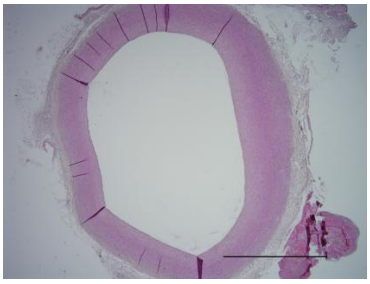


Figure 4.11: Histological micrographs of hematoxylin/eosin-stained liver, heart and coronary artery, brachial artery and aorta collected from pigs after 28 days of sham (A,C,E,G) or environmental tobacco smoke exposure (B,D,F,H). No pathological abnormalities were noted in any of these tissues.Bar = 200 μm (A,B). Bar = 2 mm (C-H).

Table 4.11: Measures of wall thickness and diameter of arteries after 28 days environmental tobacco smoke (ETS) or sham-exposure as well as 7 days benzo[a]pyrene (BAP) or vehicle injection. Luminal diameter (LD), wall thickness (WT) and luminal diameter/wall thickness (LD/WT) of aorta, brachial and coronary arteries were determined by digital morphometric analyses of hematoxylin/eosin-stained sections. Data are expressed as mean ± standard error of the mean. No significant differences were detected using unpaired t-tests with Welch's correction.

	Coronary artery			**Brachial artery**			**Aorta**		
	LD	**WT**	**LD/WT**	**LD**	**WT**	**LD/WT**	**LD**	**WT**	**LD/WT**
Sham	1.4 ± 0.06	0.2±0.02	6.2±0.5	2.5±0.10	0.4±0.02	6.5±0.3	5.5±0.3	1.1±0.1	5.3±0.6
ETS	1.4 ± 0.12	0.2±0.04	6.2±0.4	2.4±0.20	0.4±0.02	6.9±0.8	5.7±0.3	1.1±0.1	5.3±0.3
Vehicle	1.2 ± 0.09	0.3±0.03	4.7±0.2	0.2±0.03	0.2±0.04	9.0±1.4	3.3±0.3	0.6±0.1	5.7±0.6
BAP	1.32 ± 0.12	0.3±0.02	4.6±0.4	0.2±0.03	0.3±0.03	8.7±1.4	3.5±0.3	0.5±0.1	6.7±0.5

4.5. Discussion

The most important finding of this study is that exposure to daily 1-hr ETS impaired vascular endothelial function as early as 7 days with progressive impairment continuing until the end of the experiment at 28 days. However, this effect was not mimicked by intravenous administration of a high dose of the tobacco smoke constituent, BAP. Similarly, ETS exposure, but not BAP injection, altered cardiac function by increasing EDV with a trend to increasing EF and decreasing ESV, which suggests a stimulatory effect on heart function. In contrast, both ETS exposure and BAP injection resulted in systemic inflammation, increased EROD activity, increased oxidative stress and increased biological inactivation of NO which should have produced similar levels of impairment in endothelial function. However, since endothelial function was only impaired after ETS, but not BAP exposure, the connection between NO activity and endothelial function differed in the two experiments in the current study. Finally, ETS exposure, but not BAP injection, caused lung inflammation and increased the total WBC count within BAL fluid without evidence of emphysema or changes in leukocyte elastase levels. Taken together, these results suggest either that pulmonary inflammatory responses or pulmonary increases in EROD activity may be more important links to endothelial dysfunction than systemic inflammation and NO bioactivity.

We show for the first time the feasibility of using FMD in conscious healthy pigs to assess endothelial function. Flow mediated dilation use has been previously validated in conscious dogs (Puglia *et al.*, 2006) and in humans (Harris *et al.*, 2010). However, a pig model was used in this thesis because pigs are more similar to human beings in terms of diet, anatomy, aortic size and progression of CVD than rodents or dogs (Hasenkam *et al.*, 1988). The peak dilatory response found in the current study at 90s matches what is routinely used

in humans. However, the magnitude of the FMD response we determined in pigs (18%) is slightly higher than that reported in healthy dogs (7-10%) or healthy humans (10-12%). The reason for this difference in FMD magnitude is unknown, but could be due either to species differences or to the fact that FMD measurements were performed in pigs sedated with azaperone which is known to have vasodilatory properties. However, it has been shown in humans that if arteries are pre-dilated due to a variety of other interfering stimuli *(e.g.* heat, exercise), it tends to reduce their ability to further dilate and reduces, not enhances, FMD responses (Harris *et al.*, 2010). One other difference between this study and others was the use of cross sectional rather than longitudinal ultrasound views of the brachial artery to evaluate FMD. There is debate within the human literature about which method is superior (Harris *et al.*, 2010) and at least one study claims cross-sectional views are superior to the longitudinal approach (Kao *et al.*, 2003). The decrease in FMD noted over time in both sham and ETS-exposed pigs suggests that FMD decreased with age and growth. A similar finding has been reported in rats, where a more invasive version of FMD was performed on the femoral artery (Heiss *et al.*, 2008). Flow mediated dilation was lower in 20-24 week old rats (16.0%) compared to 9-10 week old rats (12.6%) in this previous study. Therefore, based on our ability to detect similar age-related changes to that reported previously plus the reproducibility of FMD values in different groups of control pigs within this thesis, we are confident that the cross-sectional FMD method used in azaperone-sedated pigs is robust and reliable as a test of endothelial function.

Similar to what was found in human beings (Peluffo *et al.*, 2009b), the exposure of pigs to ETS results in impairment of FMD. In contrast to ETS, the tobacco smoke component BAP did not cause FMD impairment. This suggests that BAP by itself is not responsible for

mediating FMD impairment. Although BAP was not administered by inhalation for safety reasons, the administered intravenous dose was several orders of magnitude greater than the amount of BAP found in cigarette smoke (Ding *et al.*, 2008). Several studies have reported that brachial artery FMD is completely NO dependent in humans (Green, 2005). Serum nitrate/nitrite levels have been used as surrogate marker for eNOS activity and NO production. It has been shown that cigarette smoke decreases eNOS activity in a dose-dependent manner in human beings (Zhang *et al.*, 2006). Moreover, free radicals within cigarette smoke react with NO to produce peroxynitrite, thereby decreasing NO bioavailability (Zhang *et al.*, 2006). In support of this, the current study found elevated serum nitrotyrosine (a by-product of peroxynitrite) levels after ETS exposure. However, nitrotyrosine was also increased after BAP injection, suggesting a similar decrease in NO bioavailability, but yet FMD was unchanged in this experiment. The lack of change in NO production accompanied by increased nitrotyrosine after chronic exposure to either ETS or BAP exposure agrees with recent studies in rats from this laboratory (Gentner and Weber, 2010b). Possibly explaining the discrepancy in FMD responses between the current ETS and BAP experiments in pig, other investigators have shown that FMD is maintained in eNOS knockout mice (Huang *et al.*, 2001). Additionally, in human beings the administration of N-G-monomethyl-L-arginine (L-NMMA), an eNOS inhibitor, does not blunt FMD response in radial artery subjected to 15 minutes of ischemia (Mullen *et al.*, 2001). In all these cases, it has been suggested that endothelium-derived hyperpolarizing factor (EDHF) compensates where NO bioavailability is impaired. A possible explanation for the results of the current study is that although NO bioavailability was impaired after both ETS and BAP exposure, FMD remained NO-dependent after ETS exposure and thus was impaired. In contrast, the

FMD response may have switched from an NO-dependent to an EDHF-mediated dilation after BAP exposure; thereby maintaining the FMD response despite decreased NO bioavailability. The difference in time of exposure (7 days versus 28 days) is clearly an important difference between the BAP and ETS experiments. Therefore, the relative contribution of NO versus EDHF in the FMD response needs to be investigated more specifically in future time-course experiments with ETS and BAP.

Endothelial dysfunction may have a negative impact on cardiac function. By causing arterial stiffness, this leads to increased peripheral resistance, increased afterload and hypertension. Thus, chronic endothelial dysfunction can result in left ventricular hypertrophy and subsequent coronary insufficiency (Verma and Solomon, 2009). Additionally, cigarette smoke exposure enhances the progression of hypertension-induced myocardial hypertrophy in spontaneously hypertensive rats (Meurrens *et al.*, 2007). Eventually, hypertrophy progresses to contractile dysfunction and heart failure. A few investigations have found that older male smokers have higher systolic blood pressure than nonsmokers consistent with increased arterial stiffness (Primatesta *et al.*, 2001), while others, paradoxically, found lower blood pressure among smokers compared to nonsmokers (Berglund and Wilhelmsen, 1975). Recent studies from this laboratory have shown that acute ETS exposure has no effect or decreases blood pressure in rats, detected using blood pressure telemetry techniques (Gentner and Weber, 2010c)**.** In contrast, chronic ETS and BAP exposure both caused an overall increase in diastolic pressure and arterial stiffness plus a failure of both systolic and diastolic pressures to dip during periods of sleep (Gentner and Weber, 2010a)**.** In the current study, FMD was impaired after ETS exposure, but no structural or histopathological changes occurred and blood pressure remained unchanged. This may indicate that the impairment of

FMD was not yet large enough to cause a detectable change in systemic blood pressure, but longer ETS exposures than 28 days may well have led to detectable changes in blood pressure. Furthermore, the sensitivity of the method used for blood pressure evaluation in this study (oscillometric blood pressure cuff) and the small sample size could also be the cause of no change in blood pressure. The use of telemetry is now considered the gold standard method for blood pressure monitoring in rodent experiments, particularly if the expected treatment effect is <30 mmHg (Kurtz *et al.*, 2005; Pickering *et al.*, 2005), but this method requires surgery and is not routine in larger animals such as the pig. In the current study, the observed increase in EDV and ESV over time in all pigs regardless of treatment, in the absence of structural or pathological changes, is related to growth. Since we were able to detect consistent growth-related changes in cardiac function, we are confident of our ability to have detected treatment effects on cardiac function. Therefore, the lack of effect of 7-day BAP exposure on cardiac function seems real and suggests other components of ETS are responsible for the observed alterations in function or that 7 days exposure was insufficient to produce an effect. In support of the former possibility, acute nicotine (a constituent of tobacco smoke) administration is known to increase heart rate and ejection fraction in humans (Benowitz and Gourlay, 1997) and dogs (Ilebekk and Lekven, 1974). Therefore, the increased EDV, increased EF and decreased ESV observed after ETS exposure in the current study is most likely due to the acute effects of nicotine.

Hemoglobin levels are reported to be 0.3-4% higher in smoker compared to nonsmokers (Andrews and Tingen, 2006), possibly due to effects of chronic hypoxia. In this study, no significant difference in TotalHb or CO-Hb levels were found after ETS exposure despite slightly lower levels of O_2 and high levels of CO in the exposure chamber. This seems to

indicate that CO is not responsible for the observed FMD impairment. Although the significant decrease in OxyHb after ETS exposure observed in the current study may relate to the lower O_2 in the exposure chamber, similar decreases in OxyHb have also been reported in human epidemiologic studies (Van Tiel *et al.*, 2002). Therefore, the decreased hemoglobin-oxygen content may instead be a common effect of tobacco smoke exposure. By definition, MetHb is the oxidized form of iron in hemoglobin and levels increase in the presence of oxidative stress caused by smoke exposure (Wright *et al.*, 1999). Thus, the significant increase in MetHb levels after BAP exposure observed in the current study is consistent with the increased oxidative stress suggested by higher nitrotyrosine. In contrast, ETS caused a paradoxical decrease in MetHb levels, but yet nitrotyrosine was increased in the current study. Possibly related is the observation in a previous study that MetHb increased after oral BAP exposure in CYP1A1 knockout mice, but decreased in wild-type mice (Uno *et al.*, 2004). Thus, the differential expression of CYP1A1 between the ETS and BAP experiments in the current study may have led to opposite MetHb effects.

The metabolism of BAP is mediated through the enzyme CYP1A1 which converts BAP to more toxic metabolites *i.e.* 9,10-epoxy-7,8-dihydrodiol and quinone metabolites (Wislocki *et al.*, 1976). These metabolite are implicated in cancer development and oxidative stress, respectively (Wislocki *et al.*, 1976). Similar to previous reports in humans (Anttila *et al.*, 2001), the inhalation exposure of pigs to ETS resulted in CYP1A1 induction within the lung. Since the lungs are the point of entry for these ETS exposures, it seems logical that all Ahr agonists within ETS were metabolized within the lungs without escaping into the systemic circulation since liver CYP1A1 activity was unchanged. In contrast, the induction of CYP1A1 in the liver, but not the lung, after intravenous BAP exposure is expected after

intravenous injection (Wislocki *et al.*, 1976) since the liver would be the major site of detoxification for systemically absorbed Ahr agonists. The increased CYP1A1 activity within both tissues would have produced a large amount of reactive PAH metabolites, increasing oxidative stress within that tissue. However, it is unclear whether these metabolites and subsequent ROS would have lasted long enough to escape the tissue. The increased level of plasma nitrotyrosine after both BAP and ETS exposure in the current experiment suggests that some of these molecules did escape the liver and lungs, respectively, to exert effects within the systemic circulation. However, since FMD impairment was observed only after ETS, not BAP exposure, a more indirect effect, perhaps involving the lung may be more physiologically important.

Alternatively, increased systemic oxidative stress could be caused by systemic inflammation (Taddei *et al.*, 2006). C-reactive protein is a marker for systemic inflammation produced by the liver and is elevated in smokers (Antoniades *et al.*, 2004; May and Wang, 2007). Small increases in serum CRP are also used as a predictor for CVD such as coronary heart disease (Antoniades *et al.*, 2004; May and Wang, 2007). The finding that ETS caused an increase in CRP serum levels is consistent with other reports indicating that ETS causes low-level systemic inflammation (Gan *et al.*, 2005). The increase in CRP after BAP exposure indicates not only that BAP can also cause systemic inflammation, but also suggests that BAP and related compounds may be the component within cigarette smoke that is responsible for CRP elevation. Furthermore, increased WBC count observed in the current study in BAL fluid from pigs exposed to ETS indicates that ETS causes lung inflammation. The majority of inflammatory cells noted on BAL fluid smears and on histological lung section were macrophages. These cells are not only capable of generating ROS, but can also

release inflammatory cytokines that cause endothelial dysfunction (Fujii *et al.*, 2002). Lung inflammation was not evident after BAP exposure in the current study because BAP was not administered by inhalation; therefore lung-specific changes were not expected. Of all the end-points examined in the current study, FMD impairment and lung inflammation are two prominent changes that occurred with ETS, but not BAP exposure. Thus, although we cannot say for sure whether ETS-induced lung inflammation is related to FMD impairment, it seems a likely candidate that needs to be explored in future studies.

People with COPD have impaired lung function and are at increased risk of developing CVD (MacNee *et al.*, 2008). This association between COPD and CVD could be related to common risk factors such as smoking (Malerba and Romanelli, 2009). Smoking not only causes lung inflammation and oxidative systemic stress, but also results in increased release of proteolytic enzymes within the airspaces leading to emphysema (MacNee *et al.*, 2008). In the current study, likely due to the relatively short duration of exposures, no significant difference was found in BAL fluid leukocyte elastase concentration after either ETS exposure or BAP injection. Therefore, although lung inflammation was evident in the current study after 28 days of ETS exposure, it had not yet progressed to more permanent structural changes within the lungs.

In summary, this study provides a good model utilizing FMD to study changes in endothelial function due to known cardiovascular risk factors such as ETS exposure. The critical observations of the current study were that ETS exposure caused impaired endothelial function with evidence of systemic and lung inflammation, increased lung CYP1A1 activity and oxidative stress. In contrast, BAP failed to alter endothelial function despite systemic inflammation and increased oxidative stress. Taken together, these results suggest either that

pulmonary inflammatory responses or pulmonary increases in CYP1A1 activity may be more important links to endothelial dysfunction than systemic inflammation and NO bioactivity.

5. Failure of resveratrol to ameliorate pulmonary inflammation, CYP1A1 activity, and endothelial dysfunction after environmental tobacco smoke exposure in juvenile pigs

5.1. Introduction

The adverse health effects of cigarette smoke involve almost every organ system in smokers (Alberg, 2008; Bartal, 2001). However, cardiovascular and respiratory effects are most prevalent, and includ diseases such as atherosclerosis, coronary artery disease, asthma, and COPD (Armani *et al.*, 2009; Bhalla *et al.*, 2009). Exposure to second-hand tobacco smoke, also referred to as ETS, poses an increased risk for many of these same diseases. In fact, smoking with ETS exposure is considered the number one preventable risk factor contributing to CVD.

Endothelial cells play a key role in the regulation of vascular tone and impairment of normal endothelial function is thought to be an early step in the development of all CVD, including that in smokers (Luscher, 1994). Therefore, assessing endothelial function has become increasingly important as a prognostic clinical tool and a method used in mechanistic investigations of cardiovascular pathophysiology (Harris *et al.*, 2010). Flow mediated dilation is an endothelium-dependent ultrasound technique which measures the ability of conduit arteries to relax and expand in response to a shear stress and hypoxia. The extent of flow mediated dilation has been used as an early predictor for CVD (Martin, 2009) and is thought to be mediated largely by NO. Flow mediated dilation has been shown to be impaired in smokers, which might be related to an oxidative stress-mediated decrease in NO

bioavailability (Celermajer *et al.*, 1996; Karatzi *et al.*, 2007; Peluffo *et al.*, 2009b). However, the effect of ETS on endothelial function is less clearly established.

Cigarette smoke-induced oxidative stress is a major pathway contributing to the development of CVD (Minicucci *et al.*, 2009; Vardavas and Panagiotakos, 2009). Of the more than 4000 compounds found in cigarette smoke, numerous classes of toxic chemicals have been implicated in causing this increased oxidative stress including: reactive metabolites of PAHs via cytochrome P450 1A1 (CYP1A1) activity (Borgerding and Klus, 2005; Ding *et al.*, 2008), carbon monoxide (CO) (Thom *et al.*, 1997) and nicotine (Rahman and Laher, 2007). With increased oxidative stress, peroxynitrite is produced as a result of the interaction between superoxide anion and NO (Ischiropoulos and al-Mehdi, 1995; van der Vliet *et al.*, 1995) which leads to reduced NO bioavailability and impaired vasodilation (Peluffo *et al.*, 2009b). Peroxynitrite formation can be indirectly monitored by measurement of serum 3-nitrotyrosine levels, a by-product of peroxynitrite's interaction with proteins. Additionally, epidemiologic studies have shown that cigarette smokers have higher levels of CRP in their serum compared to non smokers (Antoniades *et al.*, 2004; Hanyu *et al.*, 2009). C-reactive protein is an acute phase protein elevated during systemic inflammation (Antoniades *et al.*, 2004). Individuals with high CRP serum levels exhibit increased oxidative stress (van der Vaart *et al.*, 2004) and are at increased risk of developing CVDs (May and Wang, 2007). Previous studies have also indicated that ETS exposure causes lung inflammation (see Chapter 2 of this thesis; Gentner and Weber, 2010c). Not only is this increased lung inflammation linked to lung dysfunction (Chan-Yeung and Dimich-Ward, 2003; Salvi and Barnes, 2009) and eventual development of emphysema (Sharafkhaneh *et al.*, 2008), but it may also serve as a source of systemic inflammatory mediators that

increases systemic oxidative stress. Therefore, the mechanisms by which ETS increases oxidative stress are not clear, but may involve direct or indirect actions of toxic smoke constituents as well as inflammatory reactions.

Natural antioxidants within fruits and vegetables are receiving increasing attention in recent years, because of their putative protective role in CVDs, cancer, and inflammation (Kode *et al.*, 2008). Resveratrol is a polyphenolic compound, found in the skin and seeds of red grapes, with antioxidant (Kawada *et al.*, 1998), anti-inflammatory (Donnelly *et al.*, 2004), and anti-carcinogenic properties (ElAttar and Virji, 1999). In the early nineties, an epidemiologic study reported that the low mortality among the French population from coronary heart disease compared to North Americans, despite much higher smoking rates, was related to the consumption of red wine (Renaud and de Lorgeril, 1992). The cardio-protective property of red wine has been attributed to RES. Additionally, it has been shown that RES attenuates cigarette smoke-induced oxidative stress and proinflammatory phenotypic alterations within endothelial cells (Csiszar *et al.*, 2008).

We hypothesized that chronic exposure to ETS would result in lung and systemic inflammation which in turn would result in endothelial dysfunction, impaired FMD, increased blood pressure, impaired left ventricular function and increased broncho-alveolar lavage elastase associated with emphysema. We also hypothesized that these effects could be abolished by RES treatment. In order to evaluate this hypothesis we randomized 16 castrated male pigs into 4 groups in a 2x2 factorial study design i.e. sham, RES, ETS, and RES+ETS for 14 days. A fourteen day exposure was chosen based on a previous time-course study from this laboratory showing consistent impairments in FMD after daily ETS exposure by this time-point (see Chapter 2 of this thesis). Flow mediated dilation, left ventricular end-systolic

volume (ESV), end-diastolic volume (EDV), ejection fraction (EF), mean arterial pressure, systolic pressure, diastolic pressure, CO-Hb, oxyhemoglobin (OxyHb), MetHb, totalhemoglobin (TotalHb), pO_2, pCO_2, serum nitrate/nitrite (NOx), nitrotyrosine and CRP, and liver CYP1A1 and lung CYP1A1 were measured. Total and differential cell counts, and neutrophil elastase levels in bronchalveolar lavage (BAL) fluid were performed at Day 14. Histological and morphometric analyses were performed on the abdominal aorta, brachial, and coronary arteries as well as histopathological evaluation of the lung, liver and heart.

5.2. Materials and methods

5.2.1.Animals and experimental design

All protocols were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Council at the University of Saskatchewan. Sixteen castrated male pigs (10-12 kg) were obtained from Prairie Swine Center (Saskatoon, SK) and randomized into 4 groups i.e. sham, RES, ETS and RES+ETS (4 pigs/group). The pigs were group-housed with each treatment group kept in separate pens under a 12 hr dark/12 hr light cycle. The pigs were fed normal pig starter chow (Federated Co-Operatives Ltd, Saskatoon, Canada) and water *ad libitum* except during exposure, injection or cardiovascular assessment.

5.2.2.Environmental tobacco smoke (ETS) and resveratrol (RES) exposure

A single cigarette manual smoking machine from CH Technologies Inc (Westwod, USA) was used to generate ETS (mainstream plus side stream smoke). The machine was adjusted to a rate of 3 puffs /minute with 57 ml/puff of 2 second duration. The ETS, mixed with unfiltered indoor air, was then pumped into a 500 gallon polyethylene plastic water tank modified to include inflow and out flow ports and a sealed, removable plexiglass door in which unsedated, unrestrained pigs (n=4 per group) were exposed. Pumps controlling inflow were set at 6 L/min. A total of 12 cigarettes (Canadian Classics, Rothmans, Benson & Hedges, Canada) were burned over the span of 1 hr every day for 14 days. Sham exposed and RES+sham-treated pigs (n=4 pigs/exposure) were placed in the same chamber for the same duration but with unlit cigarette attached to the smoking machine. The total particulate

count in the chamber was assessed for the cigarette exposed and control pigs using a SKC constant airflow pump (Universal 224-PCXR, Eighty Four, PA) fitted with pre-weighed mixed cellulose ester filter (0.08 μm, SKC Inc., Eighty Four, PA). Total particulates were sampled continuously for one hour at 2L/min. Carbon monoxide levels were also monitored in both groups using a T40 Rattler CO monitor (Industrial scientific, Corp, Oakdale, USA) placed inside the chamber for the duration of the exposure. The air levels of O_2 / CO_2 within the chamber were measured using a Criticare Poet IQ multiparameter gas monitor (Criticare Systems, Inc., Waukesha, USA). Pigs in the RES+sham and RES+ETS groups received a daily dose of RES (5mg/kg) (MegaRES, Danbury, CT, USA) which was given orally within a gelatin capsule inserted into their pharynx for 14 days.

5.2.3.Flow mediated dilation (FMD), echocardiography, blood pressure, and blood gas evaluation

Immediately after each sham or ETS exposure, pigs were sedated by receiving an intramuscular injection of azaperone (2.2 mg/kg, Stresnil™ Merial Inc, Canada) and body temperature was measured using a rectal thermometer. Pigs were placed on left lateral recumbency. The left brachial artery was visualized using a SonoSite 180 Plus ultrasound unit with a 5.0 MHz linear array transducer (SonoSite Canada Inc., Markham, Ont., Canada). The probe was placed on the medial aspect of the distal third of the left radius, approximately 10 centimeters from the axilla. The blood pressure cuff was applied directly distal to the ultrasound area. The brachial artery was visualized at baseline (unoccluded). The blood pressure cuff was then inflated to ~30 mm Hg above systolic pressure for 4 minutes.

Following cuff release the brachial artery was visualized for 150 s. All ultrasound views were recorded using a digital video camera for the duration of the ultrasound time and uploaded to a computer. Single digital images of the brachial artery were created at baseline and 90 s, after cuff release using Adobe Premiere Elements (Adobe Inc., San Jose, CA, USA). The perimeter (P) of the brachial artery at each time point was traced and measured using Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA) and converted to a diameter using the formula: diameter = P/π . The diameter of the brachial artery at baseline and 90s were used to calculate FMD. All measurements of digital ultrasound images were performed blinded. The flow mediated dilation was calculated using the formula:

$$\% \text{ FMD} = \frac{(\text{90s post-release diameter}) - (\text{baseline diameter})}{(\text{baseline diameter})} \times 100\%$$

The left ventricular end-systolic volume (ESV) and end-diastolic volume (EDV) were measured using an HP SONOS 100CF ultrasound machine. A 5.0 MHz cardiac transducer was used in the right parasternal long-axis view to visualize the left ventricular outflow tract during systole and diastole. For each end point two measurements were taken and then averaged. The ejection fraction (EF) was calculated using the formula:

$$EF = [(EDV - ESV) / (EDV)] \times 100\%$$

After vascular and cardiac ultrasound, a Memo Diagnostic High Definition Oscillometer (S + B medVET, Markham, Ont. Canada) was placed on the distal end of the right hind limb to measure systolic pressure, diastolic pressure, mean arterial pressure (MAP) and heart rate. The mean of at least 5 consecutive blood pressure readings from each pig was used from a given session for statistical analyses. Finally, blood gases were measured in venous blood collected from the jugular vein into blood gas syringes ((Radiometer PICO 50; Radiometer;

Copenhagen, Denmark). Oxygenated hemoglobin (oxyHb), carboxy-hemoglobin (CO-Hb), methemoglobin (MetHb) and total hemoglobin (TotalHb) levels were measured using a Rapidlab 865 blood gas analyzer (Bayer Diagnostics, East Walpole, MA, USA). Ultrasound, blood pressure and blood gas measurements were taken at baseline (*i.e*. Day 0 or prior to starting the exposures), day 1, day 7, and day 14, of the experiment.

5.2.4.Plasma nitrate/nitrite, cotinine, nitrotyrosine, C-reactive protein (CRP), and ethoxyresorufin-o-deethylase (EROD) activity

Blood samples were collected from all pigs at baseline and on the last day of experiments into EDTA or serum vacutainers for plasma and serum, respectively, and stored on ice until spun at 4000xg for 10 minutes. Plasma and serum were aliquoted, then stored at -80°C until used in assays. Plasma nitrate/nitrite (NOx) levels were assessed using a commercially available enzyme-based kit (NO Quantitation Kit, Active Motif North America, Carlsbad, USA). Plasma cotinine (Bio-Quant, Inc, San Diego, USA), plasma nitrotyrosine (Cell Sciences, Canton, USA) and serum CRP (CRP; Geneway Biotech, CA, USA) levels were measured using commercially available enzyme-linked immunosorbent assays. Liver and lung homogenates were used to produce microsomes from all pigs and were then analyzed for CYP1A1 using EROD activity, as previously described (Weber *et al.*, 2002).

5.2.5.Bronchoalveolar lavage (BAL) fluid differential, total cell count and leukocyte elastase activity

At the end of each experiment pigs were euthanized by an intravenous injection with pentobarbital sodium (Euthanol; 100 mg/kg). Bronchoalveolar lavage was performed by infusing 40 ml of normal saline into the isolated trachea and lungs, with 20 ml of the lavage fluid retrieved in this process. First a hemocytometer was used to determine the total white blood cell count, then a cytospin preparation stained with Wright's solution was used to determine the differential cell counts in each BAL sample. Bronchoalveolar lavage fluid leukocyte elastase activity was measured using a synthetic substrate *i.e*. N-succinyl-$(ala)_3$-*p*-nitroanilide (Sigma-Aldrich, ON, Canada) as previously described (Castillo *et al.*, 1979). The activity of the enzyme was measured in units in which one unit of leukocyte elastase released one nanomole of *p*-nitrophenol per sec from BOC-L-alanine *p*-nitrophenyl ester at pH 6.5 at 37 °C.

5.2.6.Histological and morphometric analysis

Following euthanasia, tissues from the heart, lung, liver as well as aorta, coronary and brachial arteries were collected and placed in 10% formalin. Tissues were paraffin-embedded, serial 5 μm sections cut, stained with hematoxylin and eosin (H& E), and mounted with Micromount (Surgipath, AB, Canada). All tissues were evaluated for histopathological changes by a veterinary pathologist. For each artery, morphometric digital analyses were performed on 3 images captured from 3 different sections. Histological micrographs were taken using an Olympus BX41 microscope, attached to an Olympus DP71

digital camera, and captured using DP controller software (Olympus Canada Inc., Markham, Canada). Image Pro Plus7 was used to trace and measure the internal perimeter and wall thickness (WT) of each of the 3 images, and averages were then determined. From each perimeter the luminal diameter (LD) was calculated using the formula: diameter = P/π , and the ratio of LD to WT (LD/WT) was also determined.

5.2.7.Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA, USA) or Systat 11 (Chicago, IL, USA). In order to correct for individual variation in baseline measurements that occurred among pigs, all subsequent measurements were normalized by dividing by their corresponding baseline values from the same pig. Differences among groups were analyzed by three-way analysis of variance (ANOVA) with ETS, RES and time as factors followed by modified Bonferroni posteriori tests. Terminal end points were analyzed by a two- way ANOVA with RES and ETS as factors. A p-value < 0.05 was considered significant. All data were expressed as mean ± standard error of the mean (SEM).

5.3. Results

5.3.1. Exposure conditions, flow mediated dilation (FMD), echocardiography, blood pressure, and blood gases evaluation

Chamber conditions during ETS and sham exposures are summarized in Table 5.1. In general, the levels of CO and total particulates were higher during ETS exposures than control exposures. Chamber O_2 levels decreased after ETS exposure compared to sham exposure, but remained ≥21%. In contrast, CO_2 levels remained below the detection limit (<0.5%) during both exposures. Serum cotinine levels after 4-weeks exposure were significantly higher in ETS-exposed and ETS+RES-treated group compared to corresponding sham-exposed pigs (Table 5.1).

In order to assess the effects of ETS and RES on cardiovascular function *in vivo*, ultrasound and blood pressure assessments were performed weekly in pigs. A significant decrease in FMD was noted over time and FMD was further decreased by 7 days of ETS treatment, but with a significant interaction between the two variables (Figure 5.1). Resveratrol treatment alone or with ETS exposure had no effect on FMD. More importantly, FMD remained impaired in pigs receiving RES + ETS treatment compared to ETS treatment alone. Left ventricular end-diastolic volume (EDV), end-systolic volume (ESV) and ejection fraction (EF) increased significantly in a time-dependent manner as the pigs continued to grow (Table 5.2). ETS exposure showed no significant effect on EDV, ESV or EF, even after 14 days of treatment (Table 5.2). In contrast, ETS exposed pigs receiving RES treatment showed a significant increase in EF compared to ETS pigs (Figures 5.2 & 5.3). Additionally,

Table 5.1: Chamber conditions during environmental tobacco smoke (ETS) or sham exposures. Plasma cotinine concentrations in pigs from ETS and sham groups without and with resveratrol treatment (-RES and +RES, respectively; n=4/group) after 14 days of exposure. Temperature and carbon monoxide (CO) were continuously monitored for 1 hr during ETS or sham exposure, and were expressed as a mean of 3 readings taken every 20 minutes. Particulates were sampled continuously during 1 hr sham or ETS exposure and are expressed as total particulate matter/hr. A total of 11-12 cigarettes were burned during the daily ETS exposure or air pumped through unlit cigarettes for sham exposures. Data are expressed as mean ± standard error. *$p<0.05$ compared to sham in unpaired t-test with Welch's correction. #$p<0.05$ compared to corresponding sham group in modified Bonferroni test after two-way analysis of variance (ANOVA) with ETS and RES as factors.

	Sham	**ETS**
Temperature	24ºC	23ºC
CO (mean)	0	136 ppm
Particulates	30 μg/m3	110 μg/m3
O2	24%	21%
CO2	<0.5%	<0.5%
Cotinine -RES (ng/mL)	3.76 ± 0.36	31.27 ± 4.87*
+RES	4.21 ± 0.24	35.4 ± 4.13*

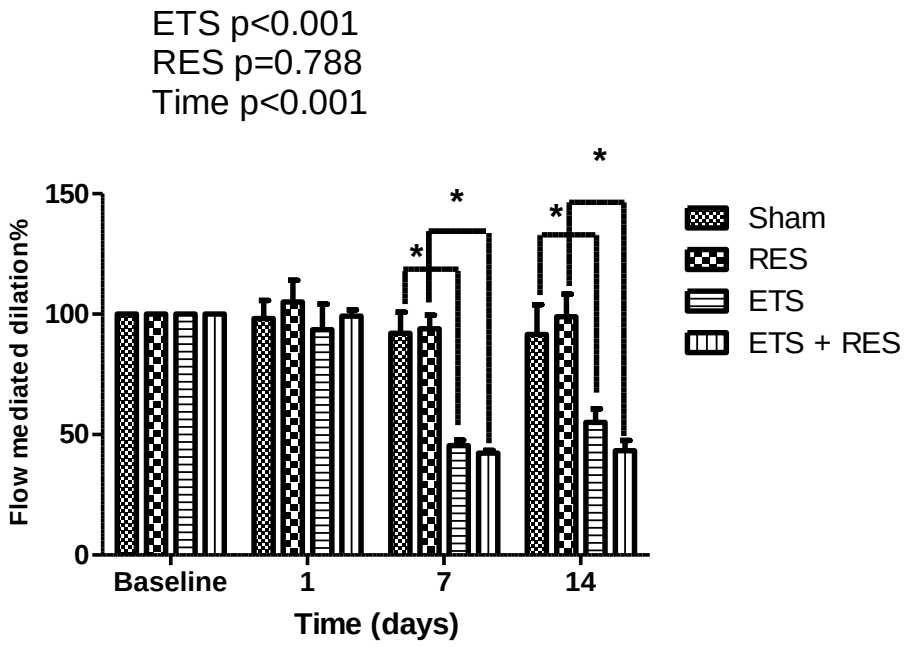


Figure 5.1 Time-course of changes in flow mediated dilation after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days. Data for each animal (n=4 per group) are expressed as a percent of the baseline (or pre-exposure) response in the same individual. Data were analyzed by three-way analysis of variance (ANOVA) with ETS, RES and time as factors, and expressed as mean ± standard errors of the means (SEM). *$p<0.05$ in modified Bonferroni posteriori test after three-way ANOVA. A significant interaction was found between ETS and time ($p<0.001$). No other interactions were found.

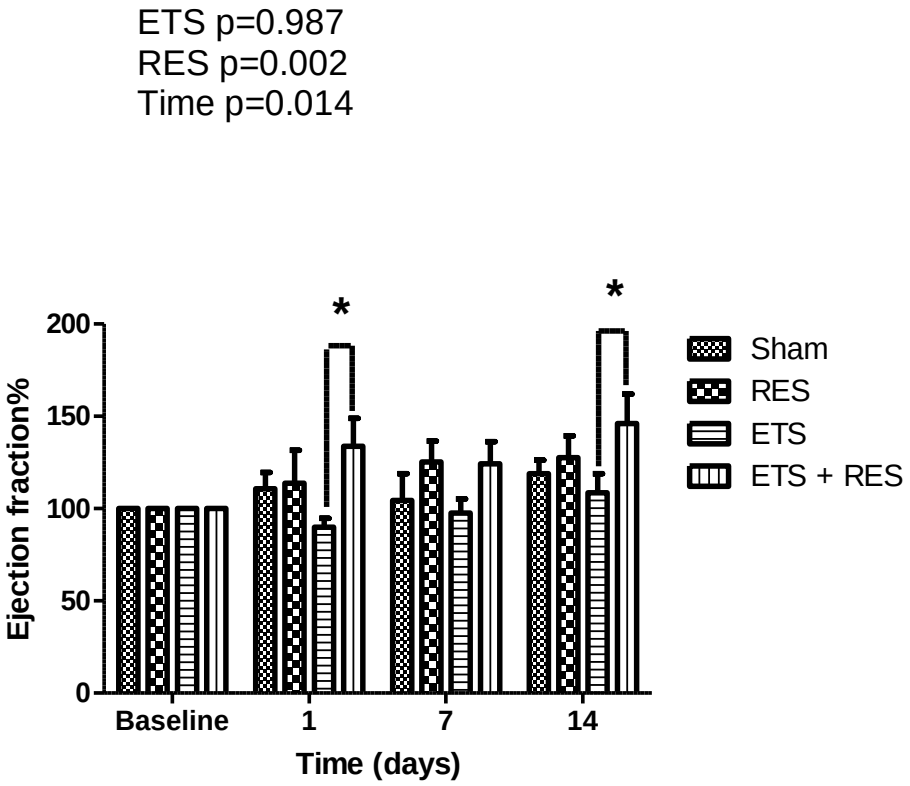


Figure 5.2 Time-course of changes in left ventricular ejection fraction (EF) after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days. Data for each animal (n=4 per group) are expressed as a percent of the baseline (or pre-exposure) response in the same individual. Data were analyzed by three way analysis of variance (ANOVA) with ETS, RES and time as factors, and expressed as means ± standard errors of the means (SEM). *$p<0.05$ in modified Bonferroni posteriori test after three-way ANOVA. No significant interactions among ANOVA factors were found.

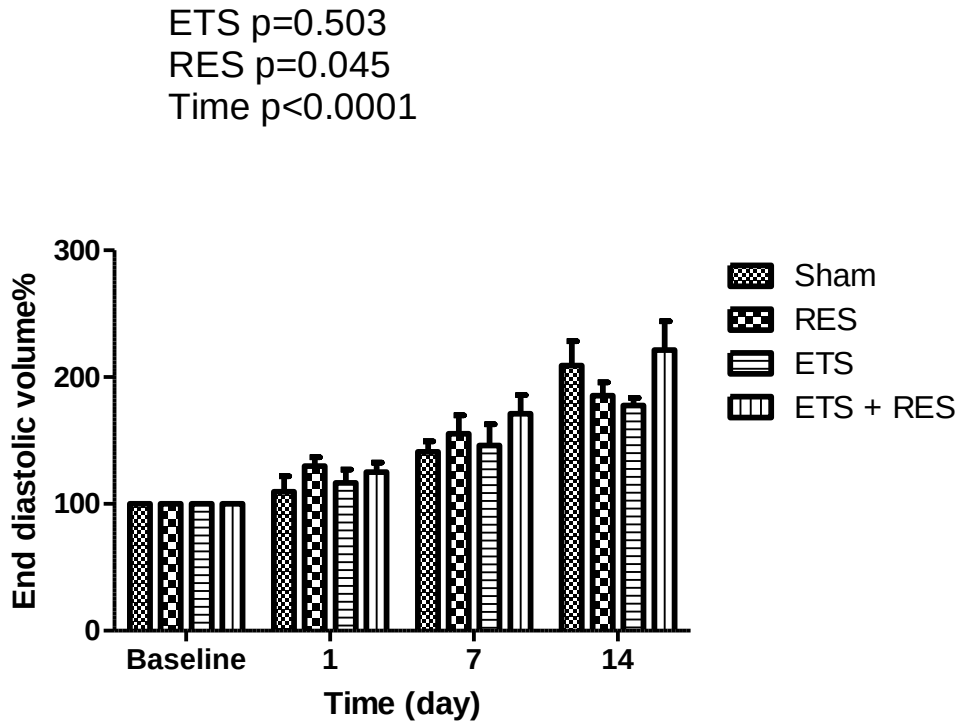


Figure 5.3 Time-course of changes in left ventricular end diastolic volume after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days. Data for each animal (n=4 per group) are expressed as a percent of the baseline (or pre-exposure) response in the same individual. Data were analyzed by three way analysis of variance (ANOVA) with ETS, RES and time as factors, and expressed as means ± standard errors of the means (SEM). *$p<0.05$ for time factor in modified Bonferroni posteriori test after three-way ANOVA. No significant interactions among ANOVA factors were found.

RES treatment resulted in a significant overall increase in EDV but had no effect on ESV (Table 5.2).

Heart and body weight were determined at the termination of the experiment. No significant difference was found in heart weight, body weight or heart/body weight ratio between all groups (Table 5.3). Blood pressure (systolic, diastolic, mean and pulse pressures) was not significantly altered over time or any treatment type (Table 5.4). In contrast, ETS exposure and RES treatment both caused a significant overall decrease in MetHb (Figure 5.4). However, neither ETS nor RES had any effect on OxyHb, CO-Hb or TotalHb (Table 5.5)

5.3.2.Plasma nitrate/nitrite, nitrotyrosine, C-reactive protein (CRP), and ethoxyresorufin-o-deethylase (EROD) activity

Overall, serum nitrate/nitrite levels (an indicator of NO production) were highly variable and no significant changes were observed over time or as a result of any treatment (Table 5.6). In contrast, ETS exposure caused a significant increase in serum nitrotyrosine levels (an indicator of NO inactivation by superoxide free radical via peroxynitrite formation) compared to sham exposure (Figure 5.5). On the other hand, RES treatment alone or with ETS exposure had no effect on nitrotyrosine levels compared to the corresponding group without RES treatment (Figure 5.5). Similarly ETS exposure resulted in a significant increase in serum CRP levels compared to sham exposure, both in the presence and absence of RES treatment (Figure 5.6). Resveratrol treatment alone had no effect on CRP levels (Figure 5.6). A significant increase in lung, but not liver EROD activity was found after ETS exposure compared to sham exposure

Table 5.2: Absolute values obtained for left ventricular end diastolic volume (EDV), end systolic volume (ESV), and ejection fraction (EF) determined over time after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

	Treatment	Baseline	Day 1	Day 7	Day 14
ESV (ml)	**Sham**	3.4±0.3	3.3±0.3	4.7±0.6	5.6±0.6
	RES	3.8±0.4	4.5±0.7	4.4±0.5	5.2±0.7
	ETS	3.5±0.2	4.5±0.3	5.1±0.3	5.8±0.5
	RES+ETS	3.8±0.4	3.4±0.3	5.2±0.7	5.3±0.9
EDV (ml)	**Sham**	7.5±0.3	8.1±0.7	10.5±0.4	15.5±0.8
	RES	7.3±0.4	9.5±0.3	11.3±0.5	13.6±0.6
	ETS	7.4±0.5	8.5±0.3	10.6±0.8	13.1±0.5
	RES+ETS	7.2±0.5	8.9±0.2	12.3±1.2	15.9±1.6
EF (%)	**Sham**	54±4	59±1	55±6	54±2
	RES	49±4	54±6	61±5	62±5
	ETS	52±3	47±2	60±4	56±3
	RES+ETS	47±5	61±3	58±4	67±3

Table 5.3: Heart weight, body weight and heart / body weight ratio after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. No significant differences were detected using a two way analysis of variance with ETS and RES as factors.

	Heart weight (Kg)	**Body weight (Kg)**	**Heart / Body weight (%)**
Sham	0.163 ± 0.013	18.19 ± 1.37	0.89 ± 0.03
RES	0.178 ± 0.004	20.94 ± 2.08	0.87 ± 0.07
ETS	0.162 ± 0.015	20.38 ± 3.06	0.83 ± 0.09
RES+ETS	0.177 ± 0.003	20.39 ± 1.42	0.88 ± 0.04

Table 5.4: Absolute values for systolic, diastolic, mean arterial and pulse pressure (mmHg) over time after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were all performed on data normalized to pre-exposure values and results with significant changes are shown in corresponding figures.

`	Treatment	Baseline	Day 1	Day 7	Day 14
Systolic pressure (mm Hg)	**Sham**	120±9	110±14	133±7	137±8
	RES	135±10	126±5	129±6	127±14
	ETS	123±11	127±11	118±21	126±12
	RES+ETS	121±11	140±14	127±14	139±10
Diastolic pressure (mm Hg)	**Sham**	59±8	52±3	56±3	55±3
	RES	60±1	63±2	55±3	56±3
	ETS	56±3	60±5	57±4	54±4
	RES+ETS	61±5	58±7	52±4	57±4
Mean arterial pressure (mm Hg)	**Sham**	81±8	72±6	83±3	83±4
	RES	87±3	86±3	81±3	81±7
	ETS	79±5	83±7	79±10	79±6
	RES+ETS	82±6	86±9	79±6	86±6
Pulse pressure (mm Hg)	**Sham**	61±7	58±12	77±8	82±5
	RES	75±11	63±3	74±6	71±11
	ETS	67±10	68±7	61±18	72±10
	RES+ETS	60±9	83±10	75±11	82±7

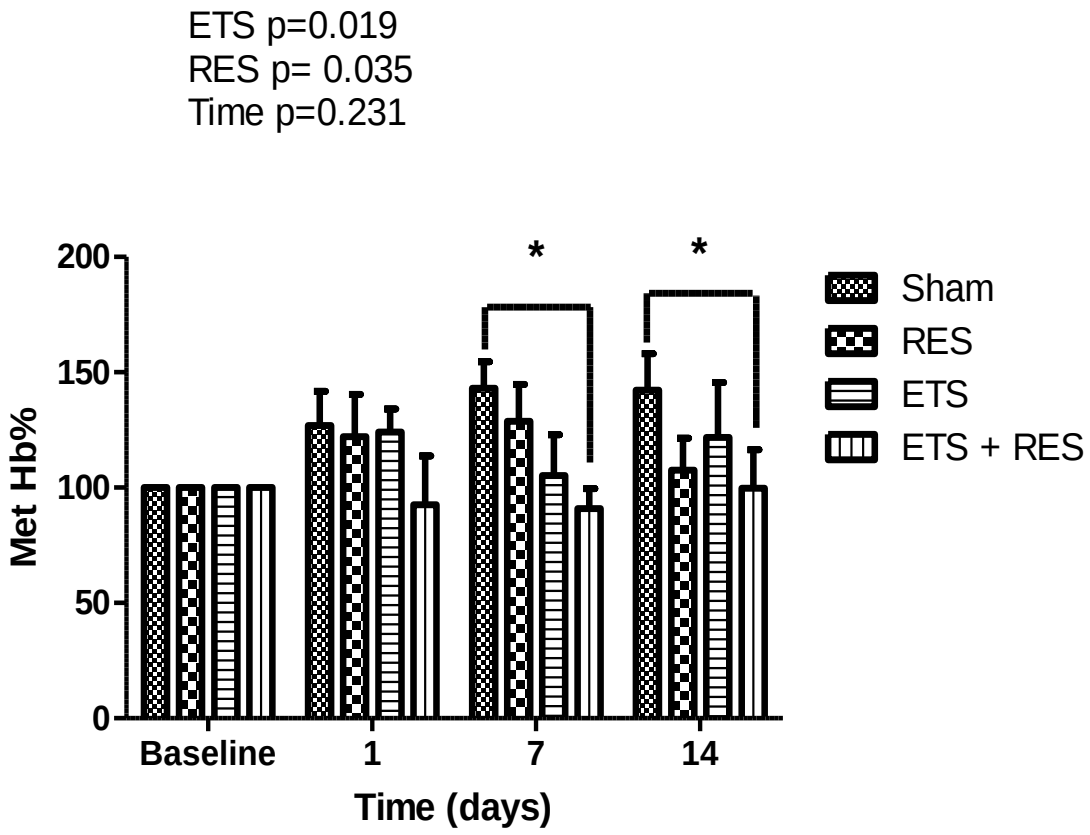


Figure 5.4: Time course of changes in methemoglobin (MetHb) levels after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days. Data for each animal (n=4 per group) are expressed as a percent of the baseline (or pre-exposure) response in the same individual. Data were analyzed by three way analysis of variance (ANOVA) with ETS, RES and time as factors, and expressed as means ± standard errors of the means (SEM). *$p<0.05$ by Bonferroni posteriori test after three-way ANOVA. No interactions were found.

Table 5.5: Time course of change in venous blood oxyhemoglobin (OxyHb) carboxy-hemoglobin (CO-Hb), methemoglobin (MetHb) and total hemoglobin (TotalHb) after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were performed on data normalized to pre-exposure values and results with significant changes are shown in the corresponding figures.

	Treatment	Baseline	Day 1	Day 7	Day 14
OxyHb (%)	**Sham**	88±7	72±8	64±5	75±6
	RES	72±11	73±7	74±5	73±7
	ETS	86±5	76±6	69±8	76±10
	RES+ETS	86±8	67±5	73±2	74±8
CO-Hb (%)	**Sham**	0.13±0.03	0.08±0.05	0.08±0.03	0.05±0.03
	RES	0. 10±0.00	0.10±0.00	0. 10±0.00	0.09±0.04
	ETS	0.10±0.00	0.08±0.03	0.13±0.08	0.05±0.03
	RES+ETS	0.08±0.03	0.13±0.03	0.13±0.03	0.08±0.03
MetHb (%)	**Sham**	1.85±0.01	2.35±0.29	2.63±0.16	2.60±0.21
	RES	2.30±0.24	2.68±0.17	2.85±0.10	2.42±0.27
	ETS	2.18±0.18	2.65±0.65	3.30±0.45	2.53±0.31
	RES+ETS	2.45±0.41	2.13±0.42	2.13±0.17	2.28±0.19
TotalHb (%)	**Sham**	102±9	100±11	92±8	102±5
	RES	105±4	96±3	99±7	96±8
	ETS	106±2	100±7	100±4	103±10
	RES+ETS	102±9	98±3	104±4	114±5

Table 5.6: Time course of change in total plasma nitrate/nitrite level after daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment for 14 days (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. Absolute values are shown for qualitative evaluation only since statistical analyses were performed on data normalized to pre-exposure values. No statistically significant differences were detected using three-way analysis of variance (ETS, RES and time as factors).

	Total nitrate/nitrite (μM)			
	Sham	**RES**	**ETS**	**RES+ETS**
Baseline	5.12 ± 1.78	6.37 ± 1.68	5.73 ± 1.60	8.87 ± 1.79
Day 1	2.42 ± 1.02	4.10 ± 0.50	5.13 ± 1.62	5.78 ± 1.66
Day 14	5.20 ± 1.46	1.73 ± 0.57	1.77 ± 0.55	3.39 ± 0.86

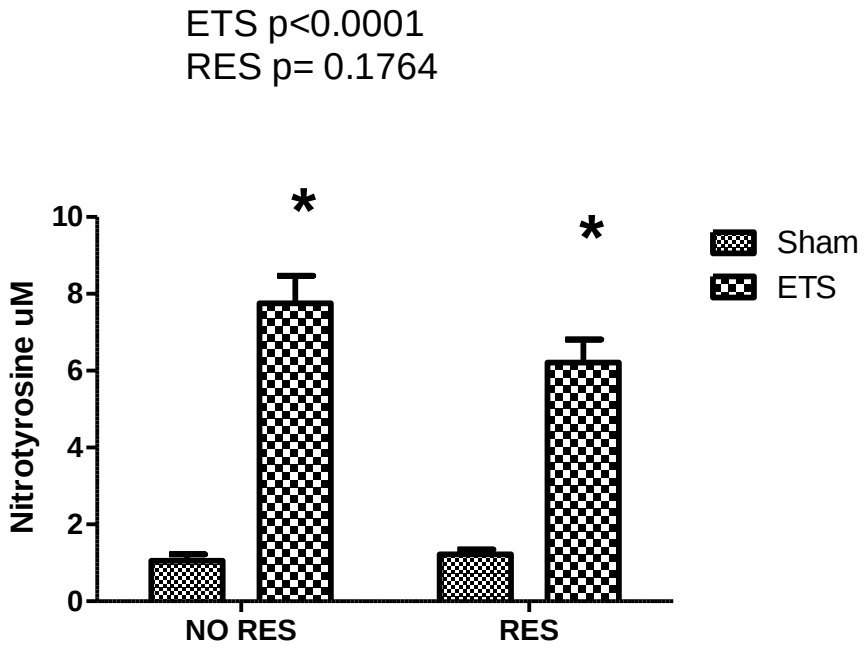


Figure 5.5: Serum nitrotyrosine levels after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. *p<0.05 in modified Bonferroni posteriori test after two-way analysis of variance (ETS and RES as factors).

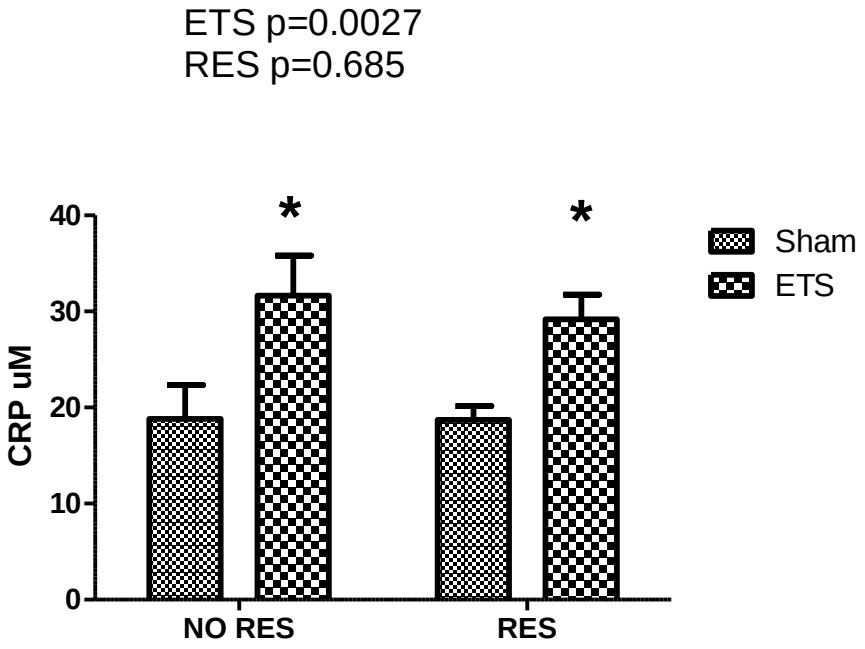


Figure 5.6: Serum C-reactive protein (CRP) levels after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Data are expressed as mean ± standard error of the mean.

(Figure 5.7). Resveratrol treatment alone had no effect on either lung or liver EROD activity and also had no effect on ETS-mediated increases in lung EROD activity (Figure 5.7).

5.3.3.Bronchoalveolar lavage (BAL) fluid total white blood cell count, BAL differential cell count, BAL leukocyte elastase activity and tissue histology

The total WBC count in BAL fluid was significantly increased after 14-days of daily ETS exposure (Figure 5.8), but not after sham exposure. Resveratrol treatment alone or in combination with ETS had no effect on BAL total WBC count (Figure 5.8). Macrophages were the only inflammatory cell type found within BAL fluid from all treatments. A qualitative evaluation of histological lung sections also showed an increase in intra-alveolar macrophages in ETS-exposed pigs compared to sham-exposed pigs, both in the presence and absence of RES, with no histological evidence of emphysema (*i.e.* no enlarged air sacs; Figure 5.9). A small, but significant increase in BAL fluid leukocyte elastase activity was found after ETS exposure in the overall two-way ANOVA analysis (Figure 5.10). However no significant differences in BAL fluid leukocyte elastase activity were found in subsequent pair-wise posteriori comparisons (Figure 5.10). No histopathological lesions were detected in any other tissue that was evaluated; liver, heart or arteries (Figure 5.11). Finally, there were no significant differences in arterial wall thickness or arterial diameter (evaluated as luminal diameter, wall thickness or a ratio of the two) in aorta, brachial artery or coronary artery among all treatment groups (Table 5.7).

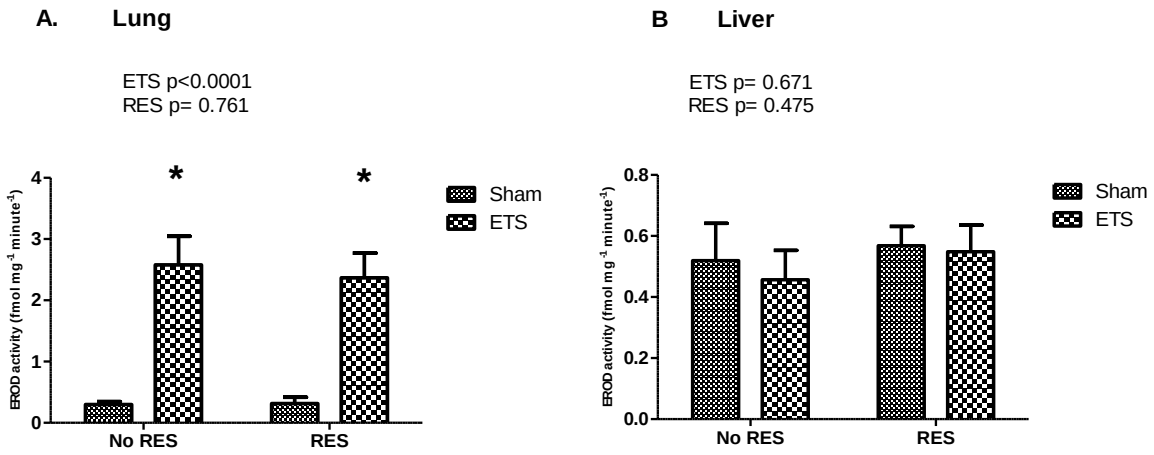


Figure 5.7: Ethoxyresorufin-o-deethylase (EROD) activity in lung (A) and liver (B) microsomes prepared from pigs after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. *$p<0.05$ in modified Bonferroni posteriori test after two-way analysis of variance.

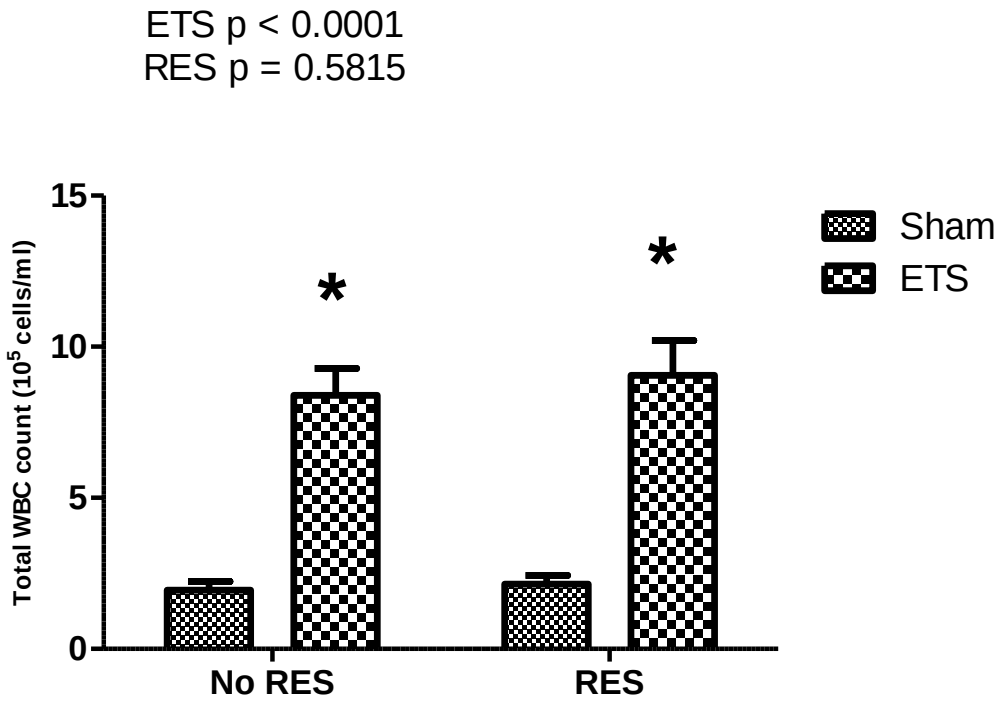
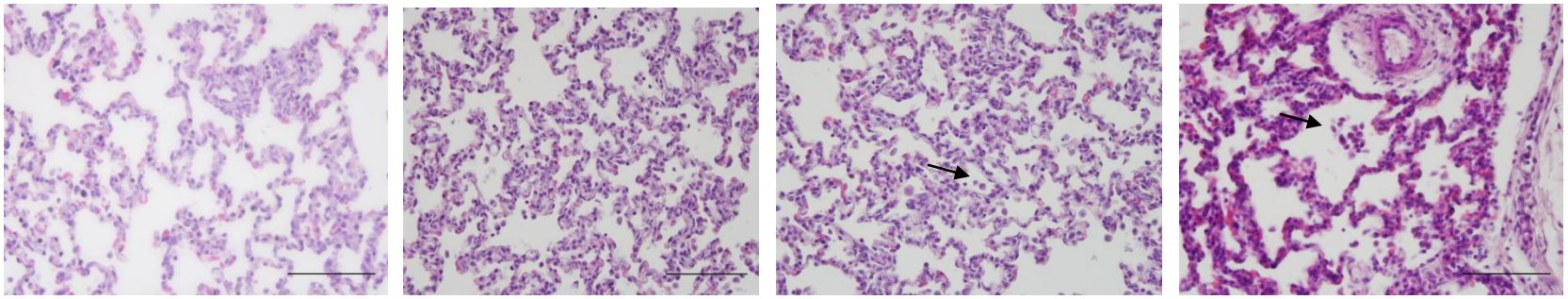


Figure 5.8: Total white blood cell count in bronchoalveolar lavage (BAL) fluid collected from pigs after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. *$p<0.05$ in modified Bonferroni posteriori test after two-way analysis of variance.



Sham RES ETS RES+ETS

Figure 5.9: Representative hematoxylin-eosin-stained lung sections from pigs after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment. Lungs from ETS-exposed pigs, both with and without RES treatment, showed increased alveolar macrophages (indicated with arrows). Bar on micrographs indicates 50 μm.

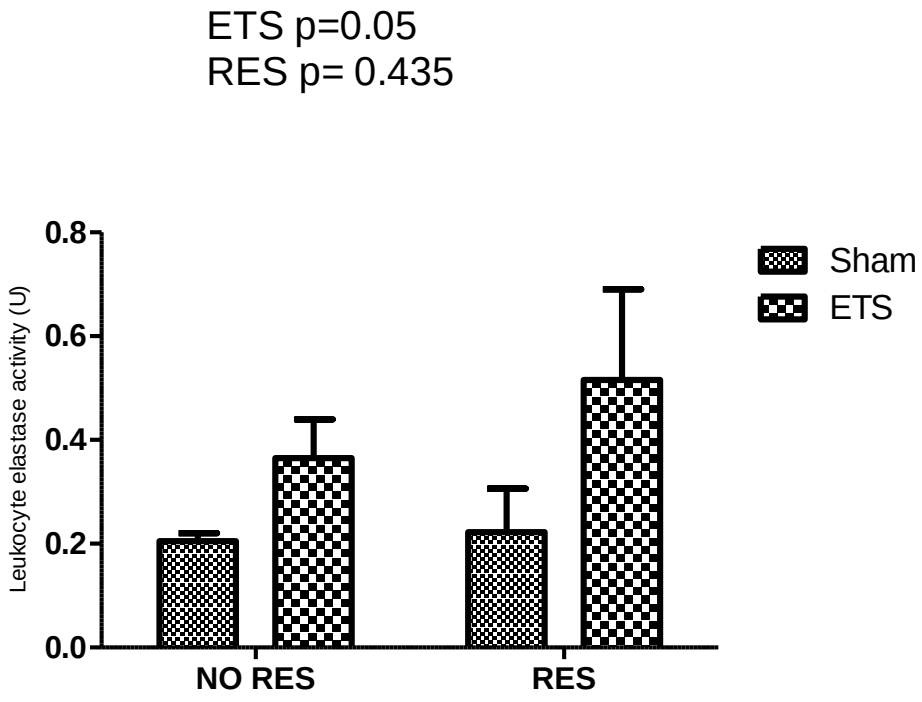
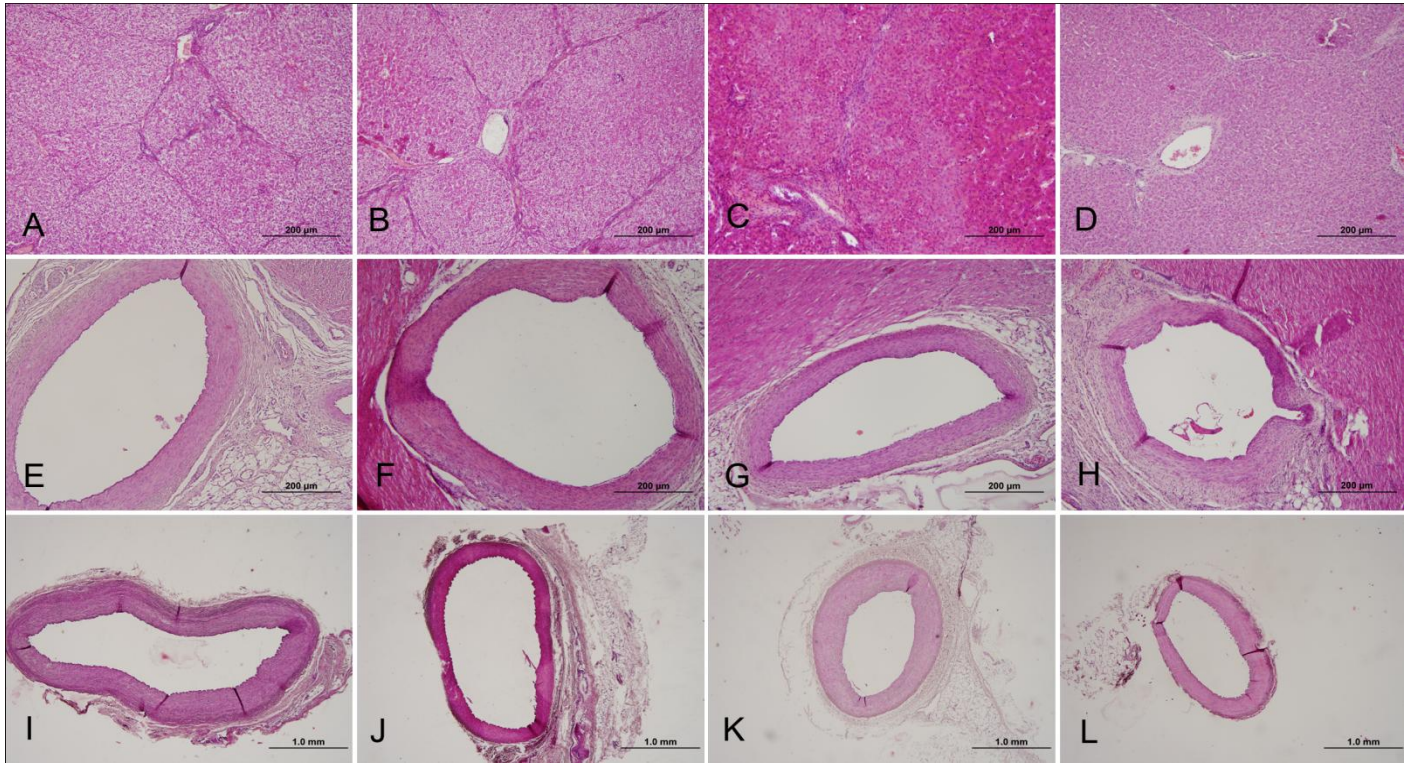


Figure 5.10: Leukocyte elastase concentration in bronchoalveolar lavage (BAL) fluid collected from pigs after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Data are expressed as mean ± standard error of the mean. *$p<0.05$ in modified Bonferroni posteriori test after two-way analysis of variance. U = one unit of leukocyte elastase releases one nanomole of *p*-nitrophenol per sec from BOC-L-alanine *p*-nitrophenyl ester at pH 6.5 at 37 °C.



Sham RES ETS RES+ETS

Figure 5.11. Representative hematoxylin-eosin-stained sections of liver (A,B,C,D), heart with accompanying coronary artery (E,F,G,H) and brachial artery (I,J,K,L) collected from pigs after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment. No histopathological lesions were observed in any of these organs in any treatment group. Bar on micrographs indicates 1.0 mm.

Table 5.7: Wall thickness and diameter of arteries after 14 days of daily 1-hr sham or environmental tobacco smoke (ETS) exposure, either with or without oral resveratrol (RES; 5mg/kg) treatment (n=4 pigs/group). Luminal diameter (LD), wall thickness (WT) and luminal diameter/wall thickness (LD/WT) of aorta, brachial and coronary arteries were determined by digital morphometric analyses of hematoxylin/eosin-stained sections. Data are expressed as mean ± standard error of the mean. No statistically significant differences were detected using separate two-way analyses of variances with ETS and RES as factors.

	Coronary artery			**Brachial artery**			**Aorta**		
	LD	**WT**	**LD/WT**	**LD**	**WT**	**LD/WT**	**LD**	**WT**	**LD/WT**
Sham	1.27±0.04	0.18±0.01	7.3±0.4	2.19±0.10	0.35±0.01	6.4±0.3	4.73±0.24	0.80±0.05	5.9±0.4
RES	1.22±0.03	0.18±0.01	6.6±0.4	2.24±0.20	0.33±0.01	6.8±0.3	4.71±0.27	0.81±0.06	5.9±0.7
ETS	1.24±0.04	0.19±0.01	6.7±0.2	2.23±0.05	0.36±0.01	6.2±0.3	4.86±0.23	0.77±0.07	6.4±0.8
RES+ETS	1.23±0.03	0.18±0.01	6.9±0.6	2.18±0.08	0.33±0.02	6.7±0.5	4.47±0.32	0.83±0.05	5.4±0.3

5.4. Discussion

The most important finding of this study is that, similar to a previous study from this laboratory, exposure to daily 1-hr ETS impaired vascular endothelial function as early as 7 days, with further impairment by 14 days. However, RES failed to reverse the ETS-mediated FMD impairment. Additionally, ETS exposure resulted in systemic inflammation, increased oxidative stress and increased biological inactivation of NO, all of which can cause endothelial dysfunction. Within the lung, ETS exposure resulted in lung inflammation, increased lung EROD activity, increased total WBC count (macrophages) in BAL fluid and increased BAL leukocyte elastase activity without histological evidence of emphysema. Finally, co-treatment with RES and ETS improved cardiac function (increased left ventricular EDV and EF) compared to ETS alone, which suggests that RES improves venous return. However, RES treatment failed to mitigate other ETS-mediated effects including systemic inflammation, oxidative stress or any effects on the lung. Taken together, these results suggest that systemic and pulmonary inflammatory responses are important links to endothelial dysfunction, and that the favorable cardiovascular effects of RES may be related to its effect on cardiac function only.

RES is a natural substance extracted from the skin and seeds of red grapes. Some studies have claimed it has anti-atherogenic and cardiovascular protective properties (Csiszar *et al.*, 2008). Although RES has high bioavailability after oral administration, it is rapidly metabolized by the liver and only trace amounts of unchanged RES are available (Walle *et al.*, 2004). Therefore, an oral dose that was in the high end of that previously shown to be non-toxic in chronic rat feedings (Rocha *et al.*, 2009) and to have beneficial effects on FMD in obese, pre-hypertensive humans (Wong *et al.*) was used to maximize the chance of

observing RES effects. However, in the current study, RES did not improve ETS-mediated decreases in FMD or reverse the ETS-induced increase in serum nitrotyrosine and CRP. This in contrast with *in vitro* findings that RES has antioxidant (Kawada *et al.*, 1998) and anti-inflammatory (Donnelly *et al.*, 2004) effects. However, others have also reported that RES is pro-oxidant (Ahmad *et al.*, 2003), promotes atherosclerosis (Wilson *et al.*, 1996) and does not reach the lung after oral administration (Berge *et al.*, 2004). Furthermore, RES failed to exert a beneficial cardiovascular effect in rats fed a normal-fat diet, while exerting a protective effect when the rats were fed high-fat chow (Wang *et al.*, 2005). Taken together, these results suggest RES may exert a protective effect in obesity and hyperlipidemia, but not after ETS exposure. Since obesity, high fat diets and ETS exposure have all been shown to increase oxidative stress, the failure of RES to mitigate ETS-mediated FMD effects suggests that RES is not providing cardiovascular protection solely via antioxidant mechanisms.

Similar to what was found in human beings (Peluffo *et al.*, 2009b), the exposure of pigs to ETS resulted in impairment of FMD. Several studies have reported that brachial artery FMD is completely NO dependent in humans (Green, 2005). Serum nitrate/nitrite levels have been used as surrogate marker for eNOS activity and NO production. It has been shown that cigarette smoke decreases eNOS activity in a dose-dependent manner in human beings (Zhang *et al.*, 2006). Moreover, free radicals within cigarette smoke react with NO to produce peroxynitrite, thereby decreasing NO bioavailability (Zhang *et al.*, 2006). In support of this, the current study found elevated serum nitrotyrosine (a by-product of peroxynitrite) levels without a change in total NO production after ETS exposure, suggesting that decreased NO bioavailability may be related to the impairment of FMD after ETS exposure in pigs.

Endothelial dysfunction may have a negative impact on cardiac function. By causing arterial stiffness, this leads to increased peripheral resistance, increased afterload and hypertension. However, no change in blood pressure was observed in the current study, disagreeing with known acute cardiostimulatory effects known for nicotine. Although nicotine is the constituent that is responsible for cigarette smoke addiction, it does not explain the chronic effects of cigarette smoke or ETS exposure (Rahman and Laher, 2007). Even in the absence of blood pressure changes, an increase in vascular tone and hemodynamic overload may still cause myocardial systolic dysfunction because the heart has to work harder to eject blood against the increased afterload (Borlaug *et al.*, 2009). Furthermore, a prolonged increase in afterload causes left ventricular hypertrophy and alteration in left ventricular extracellular matrix (Verma and Solomon, 2009) which would then contribute to diastolic dysfunction. Although several studies have provided supportive evidence that acute and chronic exposure to cigarette smoke impairs left ventricular diastolic function (Alam *et al.*, 2002; Gulel *et al.*, 2007) and enhances the progression of hypertension-induced myocardial hypertrophy in spontaneously hypertensive rats (Meurrens *et al.*, 2007), the current study failed to detect a significant ETS-mediated effect on left ventricular function. In the current study, the observed increase in EDV and ESV over time regardless of treatment, in the absence of structural or pathological changes, is clearly related to growth of the pigs. Furthermore, the echocardiographic technique used in the current study appears sufficiently sensitive since RES was found to significantly improve left ventricular function when co-administered with ETS exposure. Since RES failed to reverse ETS-mediated impairments in FMD, it is unlikely that the effect on ventricular function (improved EF and EDV) is secondary to improvements in endothelial function. Instead, RES may be

exerting a direct cardio-protective effect similar to that previously reported in humans (Penumathsa *et al.*, 2008).

Tobacco smoke contains carbon monoxide and many unstable, reactive molecules that would increase the levels of CO-Hb and MetHb, respectively, (Borland *et al.*, 1985). Possibly due to effects of elevated CO-Hb and chronic hypoxia, hemoglobin levels are reported to be 0.3-4% higher in smokers compared to nonsmokers (Andrews and Tingen, 2006). In this study, no significant differences in TotalHb, OxyHb or CO-Hb levels were found after ETS exposure despite slightly lower levels of O_2 and higher levels of CO in the exposure chamber. In addition to known vasodilatory effects of CO (Achouh *et al.*, 2008), it has been shown that endothelial cells produce peroxynitrite after CO exposure (Thom *et al.*, 1997), suggesting that CO by itself can also stimulate oxidative stress. Thus, CO does not appear to be responsible for the observed ETS-mediated FMD impairment in the current study. By definition, MetHb is the oxidized form of hemoglobin and levels have been reported to increase in the presence of the oxidative stress caused by smoke exposure (Wright *et al.*, 1999). In contrast, the results of the current experiment with ETS and RES co-exposure are consistent with another previous study that reported decreased MetHb after ETS (Borland *et al.*, 1985). The decreased MetHb result suggests lower oxidative stress, but this is inconsistent with the increased plasma nitrotyrosine observed after ETS exposure.

Polycyclic aromatic hydrocarbons are lipophilic compounds which result from the combustion of organic material (Tithof *et al.*, 2002). Many of the actions of PAHs are mediated by a ligand activated transcription factor known as the Ahr (Korashy and El-Kadi, 2006). By binding to the xenobiotic response element on DNA, this transcription factor causes transcriptional induction of dioxin-responsive genes such as the detoxification

enzyme, CYP1A1 (Korashy and El-Kadi, 2006). *In vitro* experiments have shown that RES can antagonize Ahr (Casper *et al.*, 1999), but the failure of RES to diminish ETS-mediated increases in lung CYP1A1 in the current study supports recent studies showing a lack of in vivo antagonistic effect (Bugiak and Weber, 2009; Canistro *et al.*, 2009). The metabolism of PAHs by CYP1A1 may be mechanistically important since it results in the formation of highly reactive metabolites which, in turn, can form adducts with DNA and protein (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). Additionally, PAH metabolites can be metabolized to quinone structures which can undergo redox cycling to produce ROS (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). Similar to previous reports in humans (Anttila *et al.*, 2001), the inhalation exposure of pigs to ETS resulted in CYP1A1 induction within the lung. Since the lungs are the point of entry for these ETS exposures, it seems logical that all Ahr agonists within ETS were metabolized within the lungs without escaping into the systemic circulation since liver CYP1A1 activity was unchanged. However, the possibility that reactive metabolites escaped the lung and entered the systemic circulation to increase oxidative stress requires clarification with further experiments.

Alternatively, increased systemic oxidative stress could be caused by systemic inflammation (Taddei *et al.*, 2006). C-reactive protein is a marker for systemic inflammation produced by the liver and is elevated in human smokers (Antoniades *et al.*, 2004; May and Wang, 2007), after ETS exposure in humans (Gan *et al.*, 2005), as well as in the current study after ETS exposure in pigs. Small increases in serum CRP are also used as a predictor for CVD such as coronary heart disease (Antoniades *et al.*, 2004; May and Wang, 2007). The observation that active cigarette smoking causes lung inflammation has been reported based on the presence of neutrophils and macrophages within alveoli (van der Vaart *et al.*, 2004),

and was consistent with the increased total WBC count within the BAL fluid after ETS exposure in current study. In contrast, macrophages, not neutrophils, were the only cell type observed in the BAL fluid after ETS exposure in the current experiment. Also, it is important to note that RES did not show any ability to reverse ETS-mediated lung inflammation in the current study, suggesting that RES does not exert any anti-inflammatory action in this pig ETS model. Inflammation within the airways is known to promote decreased lung function, asthma and respiratory irritation in children and adults passively exposed to ETS (Chan-Yeung and Dimich-Ward, 2003; Salvi and Barnes, 2009). Pulmonary neutrophils and macrophages not only generate ROS and inflammatory cytokines that can impair endothelial function (Fujii *et al.*, 2002), but also release extracellular matrix degrading enzymes *i.e*. elastases which contribute to lung emphysema (Sharafkhaneh *et al.*, 2008). Consistent with other studies (Lucattelli *et al.*, 2005; Maeno *et al.*, 2007) the BAL fluid leukocyte elastase activity was increased after ETS exposure in the current experiment. This suggests that ETS-mediated inflammation was sufficient to have started emphysema pathogenesis by 14 days of exposure in pigs. However, the absence of histological evidence of lung emphysema in this study also indicates that the relatively short duration (14 days) of ETS exposure had produced only non-structural and more likely reversible changes in the lung.

In conclusion, the critical observations of the current study were that ETS exposure caused impaired endothelial function with evidence of systemic and lung inflammation, increased lung CYP1A1 activity and oxidative stress. In contrast, RES failed to reverse any ETS-induced effects, but did appear to exert a direct effect on the heart that led to improved left ventricular function. Considering the high dose level of RES used in the current study, the failure of RES is unlikely to be due to insufficient levels, but rather may be due to a lack

of efficacy in pigs or in ETS-induced lung inflammation. Future experiments are needed to confirm whether this is pig-specific difference in efficacy or a more generalized ineffectiveness of RES to ameliorate ETS-mediated cardiovascular effects.

6. Discussion & Conclusion

6.1. Similarities and differences in findings between studies in this thesis

Environmental tobacco smoke exposure resulted in similar changes to the end points measured after 2 or 4 week exposure to ETS. Similar changes were noted for FMD, CRP, nitrotyrosine, liver and lung EROD, and BAL fluid total WBC counts, which indicates that ETS can have negative health effects after as little as 14 days of exposure. The reproducibility of results between the two studies also strengthens our conclusions. Differences between the two ETS exposure experiments were noted in cardiac assessment and also in BAL fluid leukocyte elastase activity. This could be due to differences in length of exposure between the two experiments. The variability could also be related to small sample sizes and technical errors during assessment. The choice of sample size was based on power analysis aimed at detecting differences in FMD. However, our ability to consistently detect changes in cardiac size with growth further emphasizes the reliability of our echocardiography assessment. Surprisingly, the BAL fluid leukocyte elastase activity was significantly elevated in the second ETS exposure experiment despite its shorter duration. It should be noted, however, that a similar trend for change was noted in first ETS exposure experiment. Thus, the differences here may related to individual variability and could thus be related to low sample numbers.

In the first experiment the differences between the length of BAP and ETS exposure may explain the absence of change in FMD after BAP exposure. However, changes in FMD were noted as early as 7 days after ETS exposure and the dose at which BAP was administered is many orders of magnitude higher than what is found in cigarette smoke. Instead, the

difference in route of exposure (inhalation versus intravenous injection) seems to indicate that lung inflammation is a key element in FMD impairment. A possible mechanism by which endothelial function is impaired preferentially by lung inflammation is discussed further in a following section.

6.2. Resveratrol's effects

Oral resveratrol administration did not reverse any of the negative effects of ETS, which indicates that RES may not possess the claimed desirable cardiovascular effects which were largely based on results from *in vitro* testing. This failure of RES may not have been due to a low dosage of RES since the dose used was at least 3-4 times higher than what is recommended by the manufacturer (100mg/day for adults). Moreover, the dose chosen for this thesis falls within the range of commonly used doses, including several studies reporting beneficial effects with similar or lower doses in rats and mice (Jin *et al.*, 2010; Lekli *et al.*, 2008; Palsamy *et al.*; Silvia *et al.*, 2007).

The effects of RES versus BAP were predicted to be opposite if both work via interaction with Ahr (antagonist and agonist, respectively). The results of this thesis do not support this hypothesis because Ahr-related changes were seen only after BAP exposure. The increase in liver EROD activity after BAP exposure indicates that its effects are mediated at least in part through activation of Ahr. The failure of RES to reverse the Ahr-mediated effects of BAP within cigarette smoke does not support the claim that RES is an Ahr antagonist. In fact, it has been shown recently that RES may actually be a partial agonist of Ahr (Bachleda *et al.*, 2010). More importantly, RES has more recently been shown to exert effects through SIRT-

1 which may be a more important mechanism of action for beneficial health effects than AhR interaction (Borra *et al.*, 2005) .

6.3. Synthesis of tobacco smoke effects on the lung and proposed mechanism of action

It is estimated that cigarette smoke contain 5 x 10^{14} free radicals / puff, and cigarette smoke exposure results in an inflammatory response within the lung characterized by the presence of neutrophils and/or macrophages within alveolar spaces. Lung components including epithelial cells, fibroblasts and resident pulmonary macrophages respond to the smoke particulate exposure by releasing a number of chemotactic factors such as IL-8, LTB-4, MIP-1 and MCP-1 which attract neutrophils and monocytes into the lung (van der Vaart *et al.*, 2004; Wright *et al.*, 2002). Experimentally, increased or unchanged numbers of neutrophils, and unchanged numbers of macrophages were reported after acute cigarette smoke exposure (Janoff *et al.*, 1983; Morrison *et al.*, 1999). On the other hand, it has been reported that BAL fluid from healthy chronic smokers contains only macrophages (Meuronen *et al.*, 2008), or both macrophages and neutrophils (Kuschner *et al.*, 1996). The difference in inflammatory cell types within the lung after cigarette smoke exposure is most likely related to the duration of exposure. Neutrophils tend to predominate in initial inflammatory reactions while macrophages predominate with time as inflammation continues. Thus, based on the fact that in this thesis the only cell type noted within the alveoli and BAL fluid after 14 and 28 day exposure was macrophages (see Figure 6.1), this pig ETS exposure model mimicked a longer-term exposure. It is possible that a neutrophilic inflammation (Figure 6.1) was present initially in this pig model, but that this was later replaced by macrophages. Future experiments examining earlier time points after daily ETS

exposure would be needed to better address this possibility versus a species-specific response unique to pigs. The inflammatory cells within the lung as well as epithelial cells contribute to oxidative stress by releasing free radicals (Sharafkhaneh *et al.*, 2008). Smoking is also known to be a risk factor for the development of alveolar emphysema (Selman, 2003). Macrophages and neutrophils can release a number of elastolytic enzymes which can degrade the extracellular matrix such as MMP-9, MMP-12 and neutrophil elastase (Churg *et al.*, 2008). Compared to healthy nonsmokers, the BAL fluid of healthy smokers contain higher levels of elastase activity (Janoff *et al.*, 1983) which could eventually lead to dilated alveoli and overt emphysema. Thus, not only is elastase involved in COPD pathogenesis, but it is also indicative of a long period of inflammation. In this thesis, we found higher elastase activity in the BAL fluid from pigs exposed to cigarette smoke compared to sham exposed pigs but without histological evidence of emphysema. Thus, the duration of exposure in the pig ETS model used in this thesis appeared to be of a moderate length, consistent with a sub-chronic response. Overall, we believe that this inflammatory response within the lung is the key pulmonary response that is responsible for producing cardiovascular responses within the systemic circulation.

Another key pulmonary response to ETS is the activation of Ahr by PAHs, which is known to play an important role in the generation of oxidative stress and in pulmonary carcinogenesis (Korashy and El-Kadi, 2006; Moorthy *et al.*, 2003). The expression of CYP1A1 is considered a specific marker for Ahr activation. Cytochrome P450 1A1 metabolites such as quinones and epoxides are implicated in the generation of oxidative stress and in carcinogenesis, respectively (Levin *et al.*, 1982). The finding of increased lung EROD activity after smoke exposure in this thesis is consistent with a critical role of PAHs

and Ahr in mediating oxidative stress within the lung via production of reactive metabolites that could be produced in sufficient quantities to exit the lung and exert systemic effects. However, the short half-life of these reactive metabolites makes this possibility less likely than alternative explanations. Instead, Ahr activation is more likely to play a direct role in regulating the inflammatory response within the lung. One study reported that the expression of IL-8 from alveolar macrophages increased after BAP exposure which could be reversed by the transfection of cells with silencing RNA against Ahr (Podechard *et al.*, 2008). Additionally, it has been shown that Ahr activation results in increased expression of the chemokine CCL1 from alveolar macrophages (N'Diaye *et al.*, 2006) and NF-κB (Kobayashi *et al.*, 2008), both of which would initiate pulmonary inflammatory responses. In this study, the increase in lung EROD activity was associated with histological evidence of inflammation *i.e.* macrophages. Based on the results of this thesis, we propose that Ahr activation by the PAH component of tobacco smoke within alveolar endothelial cells and in macrophages is responsible for initiating the inflammatory response that then spills over into the systemic circulation to cause adverse effects (Figure 6.1).

6.4. Proposed connection between pulmonary and systemic vasculature after tobacco smoke exposure

Pulmonary endothelial cells show increased expression of adhesion molecules on their surface after tobacco smoke exposure (Scott and Palmer, 2003) and this serves to facilitate the recruitment of inflammatory cells to the lung. In addition to secreting chemokines, the inflammatory cells within lung as well as pulmonary parenchymal cells secrete cytokines that may have systemic effects. Macrophages and epithelial cells exposed to cigarette smoke

release IL-1β, TNF-α, and IL-6 which are known to increase the expression of adhesion molecules on the surface of endothelial cells (Scott and Palmer, 2003). Thus, although this was not measured in this thesis work, it is also possible that smoke particulates also contribute to increased expression of adhesion molecules on pulmonary vasculature (Figure 6.1). Interestingly, it has been shown that the levels of neutrophil- and macrophage-attracting chemokines are elevated in guinea pig BAL fluid after single and repeated smoke exposure, while the levels of TNF-α are elevated after acute but not chronic exposure (Kubo *et al.*, 2005). Others also reported that repeated exposure results in an adaptive response within the lung characterized by lower levels of leukocytes within BAL fluid compared to acute exposure in pigs (Charavaryamath *et al.*, 2005) . Unlike other studies which reported the presence of macrophages and neutrophils within BAL fluid and alveoli after chronic cigarette smoke exposure, the work in this thesis found only macrophages within alveoli and BAL fluid. Additionally, pulmonary capillaries were devoid of neutrophils and other leukocytes. It would be interesting to see if such an adaptive lung immune response occurs in the pig and whether the chemokine or cytokine levels within the lung and blood would change with more prolonged ETS exposures.

Pulmonary endothelial cells may also become a source of oxidative stress. The exposure of human, bovine, and rat pulmonary endothelial cells to cigarette smoke extract resulted in superoxide anion production which could be reversed by the inhibition of NADPH oxidase (Jaimes *et al.*, 2004). We found that brachial artery FMD was impaired after smoke exposure, which indicates that cigarette smoke has negative effects on the systemic vasculature. Tobacco smoke exposure results in systemic vascular effects by one of the following mechanisms. First, free radicals within tobacco smoke and tobacco smoke

constituents, especially, within the particulate phase, can reach the systemic circulation resulting in inflammation and oxidative stress within the vasculature. Second, the inflammatory response within the lung can also spillover into the systemic circulation and contribute to endothelial dysfunction. However, once again, the short half-life of superoxide free radicals makes the possibility of ROS effects outside the lung and the former possibility seem unlikely. Therefore, based on the results of this thesis, we propose that the latter possibility of inflammatory spillover is the connection between pulmonary ETS effects and cardiovascular effects (Figure 6.1). Tobacco smoke contains many compounds that can cross from the lung into the blood stream such as nitrogen oxides, isoprene, butadiene, benzene, styrene, formaldehyde, acetaldehyde, acrolein, furan, as well as many heavy metals like cadmium arsenic and lead. Many of these compounds can cause endothelial dysfunction.

Increased systemic oxidative stress and inflammation have been implicated in the impairment of FMD through the inactivation of NO (Karatzi *et al.*, 2007). In this thesis, we found that both ETS exposure and BAP injection resulted in systemic inflammation (high CRP levels), increased oxidative stress and increased biological inactivation of NO (high nitrotyrosine) which should have produced similar levels of impairment in endothelial function. However, FMD was impaired only after ETS exposure. Therefore, these results at first do not appear to support our proposed mechanism of action for pulmonary ETS exposure effects. However, another factor that must be considered is that FMD in pigs may not be entirely NO-dependent. We hypothesize that endothelium-derived hyperpolarizing factor (EDHF) may compensate in certain cases where NO bioavailability is impaired (Figure 6.1). In support of this, EDHF has been shown to preserve endothelium-dependent dilation when NO-dependent mechanisms become impaired in animal models of congestive heart

failure (Ueda *et al.*, 2005). Thus, the FMD response may have switched from an NO-dependent to an EDHF-mediated dilation after BAP exposure; thereby maintaining the FMD response despite decreased NO bioavailability in the current thesis. However, why a similar compensation did not occur after ETS exposure in this thesis work is not clear. One explanation may be the difference in exposure time (7 days versus 28 days) which would mean that EDHF compensation for lack of NO-dependent dilation is transient. Therefore, the relative contribution of NO versus EDHF in the FMD response needs to be investigated more specifically in future time-course experiments with ETS and BAP. Alternatively, the absence of FMD impairment after intravenous BAP administration may indicate that the lung-induced inflammatory response has unique systemic effects. This is supported by the finding that nonsmoking COPD patients have impaired FMD and elevated levels of serum CRP (Pinto-Plata *et al.*, 2006). Additionally, in a study involving 39 patients who underwent lung resection, divided into three groups, nonsmokers, smokers with normal lung function and patients with COPD. The pulmonary artery endothelial cells of COPD patients developed lower relaxation in response to ADP than nonsmokers and smokers (Peinado *et al.*, 1999). An important inflammatory mediator which could be responsible for systemic effects is IL-6. Interleukin-6 has been shown to leak out from the lung to the systemic circulation after endotoxin exposure (Tamagawa *et al.*, 2009). Furthermore, IL-6 is elevated in smokers and is known to drive the expression and release of CRP from the liver. Recently it has been shown that CRP induces endothelial dysfunction and uncoupling of eNOS in vivo (Hein *et al.*, 2009). It would be interesting to see if the lung is the primary source of IL-6 after ETS exposure. If so, it would solidify the primary role of lung induced inflammation we propose in cardiovascular disease from ETS.

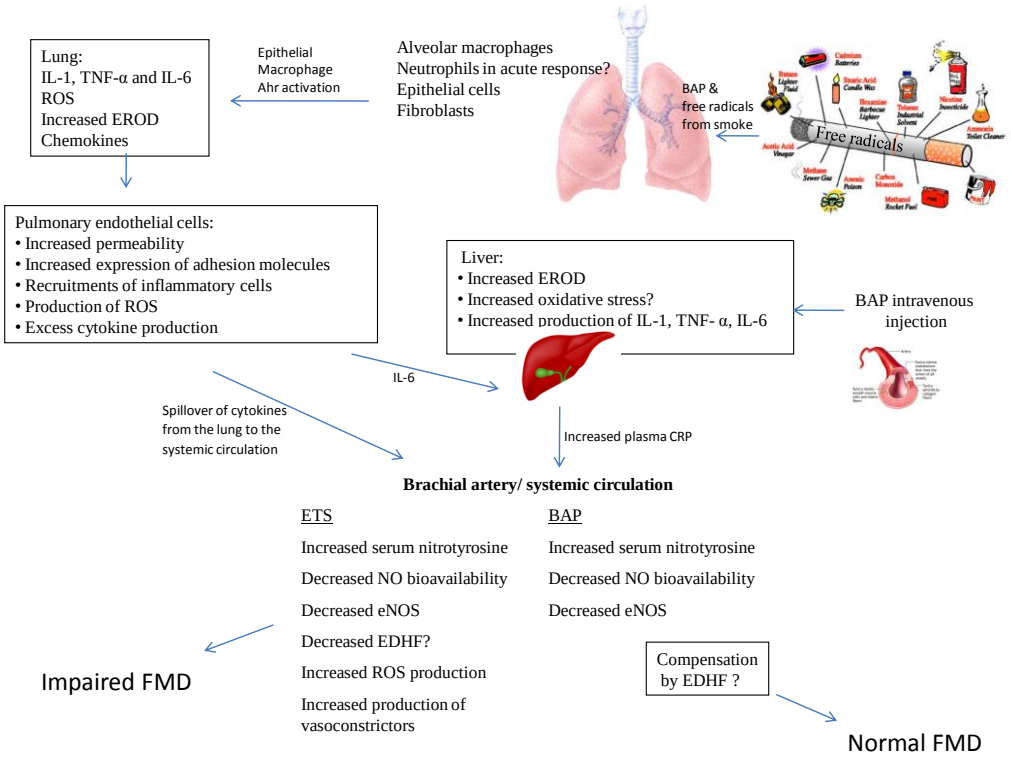


Figure 6.1: Proposed sequence of events explaining how flow mediated dilation (FMD) could be impaired after environmental tobacco smoke exposure (ETS) exposure and a possible reason for lack of impairment after intravenous benzo[a]pyrene (BAP) administration. EDHF: endothelium-derived hyperpolarizing factor. Ahr: Aryl hydrocarbon receptor. NO: nitric oxide. eNOS: endothelial nitric oxide synthase. ROS: reactive oxygen species. CRP: C-reactive protein. IL-1: Interleukin-1. TNF- α: Tumor necrosis factor- α . IL-6: Interluken-6. EROD: ethoxyresorufin-o-deethylase.

6.5. Deficiencies of studies in this thesis

The use of oscillometery for blood pressure evaluation may have failed in detecting subtle differences in blood pressure. Telemetry is now considered the gold standard approach for blood pressure evaluation. However, this method is invasive, costly, requires trained personnel and is not a standard procedure in larger animals such as pigs, but would be a logical next step for future ETS experiments in this pig model. The use of larger sample numbers to examine all the above effects will also help strengthen our conclusion.

6.6. Future experiments

The cause of FMD impairment was not established in this thesis. Therefore, future studies would focus on investigating the role of other vasodilators, such as EDHF in mediating FMD. This could be achieved by administering pharmacological blockers of EDHF, such as apamin and charybdotoxin, or a placebo to normal healthy pigs and monitoring FMD. Additionally, the role of inflammatory mediators in FMD impairment after ETS exposure should also be investigated. This could be done by measuring the levels of these mediators, such as IL-1, TNF- α and IL-6 in the systemic circulation and correlating their levels with eNOS expression, NO levels and FMD impairment after ETS exposure. It would be interesting to compare the difference in immune response in the lung after single and repeated exposures to ETS. This may shed some light on the adaptation process that may occur in the lung after repeated exposure. Finally, the beneficial cardiovascular effects of RES should be further examined using a wider range of doses, especially in whole animal models rather than *in vitro.*

6.7. Significance of thesis work: Social and health significance of environmental tobacco smoke and resveratrol

Cigarette smoking is known to impact the health of smokers affecting almost every organ system. Although there is increasing evidence linking exposure to ETS in non-smokers to several disease processes, the link is often controversial. The claim that ETS exposure has no, or at most, a minor health impact is based on ETS exposure being intermittent. Thus, the argument is that ETS yields only low and subclinical toxic exposures in non-smokers. However, almost all regulations govern smoking in public places and virtually no regulations govern cigarette smoking in private places. This claim does not take into consideration the repetitive nature of ETS exposure in private spaces such as the home, particularly when a family member smokes indoors. Additionally, the impact of ETS exposure on people with other pre-existing cardiovascular and pulmonary diseases is not clear, but is likely underestimated. Results from this thesis strongly indicate that people at risk of CVD develop a clinically measurable impairment in flow mediated dilation even after short, discontinuous exposures to ETS. Pigs provide a valuable model to study cardiovascular diseases as they are more similar to human beings in terms of diet, anatomy, aortic size and progression of CVD than rodents or dogs. The pigs used in the ETS exposure experiments were weaning age. Thus, such exposure mimics a scenario of a smoking parent causing negative health effects to his/her 1-2 year old children. Since there are no laws governing smoking in private places such as the home, this represents a relevant and common exposure scenario.

Dietary supplements are widely used because of their claimed health benefits. Unlike prescribed medications, which are known to have specific targets affecting a particular

disease pathway, the advertised health effects of dietary supplements are often more general and poorly defined. Claims of preventing or improving a wide variety of disease processes as well as increasing life span are common. Additionally, until recently, the manufacturing of dietary supplements was subject to limited oversight. Although the regulations and monitoring of manufacturing supplements have improved in recent years, critics still point out that less rigorous regulations for dietary supplements are implemented than those of drug counterparts. Resveratrol is a natural food supplement found in the skin of red grapes as well as other food items. The advertised health benefits of RES were first focused on the cardiovascular system with studies linking its consumption to decreased incidence of several CVDs. In recent years, the claimed health benefits of RES have been expanded to include cancer prevention and treatment as well as increasing life span. While many studies have reported beneficial effects of RES *in vitro,* the *in vivo* effects are far from being clear at this stage with quite a few studies challenging its health benefits. The results from this thesis indicate that RES consumption is not protective for the CVD under the conditions assessed here.

7. References

Achouh, P. E., Simonet, S., Fabiani, J.-N. and Verbeuren, T. J. (2008). Carbon monoxide induces relaxation of human internal thoracic and radial arterial grafts. Interact CardioVasc Thorac Surg, 7, 959-962.

Ahmad, K. A., Clement, M. V. and Pervaiz, S. (2003). Pro-oxidant activity of low doses of resveratrol inhibits hydrogen peroxide-induced apoptosis. Ann N Y Acad Sci, 1010, 365-373.

Alam, M., Samad, B. A., Wardell, J., Andersson, E., Hoglund, C. and Nordlander, R. (2002). Acute effects of smoking on diastolic function in healthy participants: studies by conventional doppler echocardiography and doppler tissue imaging. J Am Soc Echocardiogr, 15, 1232-1237.

Alberg, A. J. (2008). Cigarette smoking: health effects and control strategies. Drugs Today (Barc), 44, 895-904.

Ambrose, J. A. and Barua, R. S. (2004). The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol, 43, 1731-1737.

Anderson, M., Johnson, D. and Batal, H. (2005). Sudden Infant Death Syndrome and prenatal maternal smoking: rising attributed risk in the Back to Sleep era. BMC Medicine, 3, 4-5.

Andrews, J. O. and Tingen, M. S. (2006). The effect of smoking, smoking cessation, and passive smoke exposure on common laboratory values in clinical settings: a review of the evidence. Crit Care Nurs Clin North Am, 18, 63-69.

Antoniades, C., Tousoulis, D., Vasiliadou, C., Marinou, K., Tentolouris, C., Ntarladimas, I. and Stefanadis, C. (2004). Combined effects of smoking and hypercholesterolemia on

inflammatory process, thrombosis/fibrinolysis system, and forearm hyperemic response. Am J Cardiol, 94, 1181-1184.

Anttila, S., Tuominen, P., Hirvonen, A., Nurminen, M., Karjalainen, A., Hankinson, O. and Elovaara, E. (2001). CYP1A1 levels in lung tissue of tobacco smokers and polymorphisms of CYP1A1 and aromatic hydrocarbon receptor. Pharmacogenetics, 11, 501-509.

Armani, C., Landini, L., Jr. and Leone, A. (2009). Molecular and biochemical changes of the cardiovascular system due to smoking exposure. Curr Pharm Des, 15, 1038-1053.

Bachleda, P., Vrzal, R. and Dvorak, Z. (2010). Resveratrol enhances NK cell cytotoxicity: Possible role for aryl hydrocarbon receptor. Journal of Cellular Physiology, 225, 289-290.

Bartal, M. (2001). Health effects of tobacco use and exposure. Monaldi Arch Chest Dis, 56, 545-554.

Beckman, J. S. and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol, 271, C1424-1437.

Becquemin, M. H., Bertholon, J. F., Attoui, M., Roy, F., Roy, M. and Dautzenberg, B. (2007). Particle size in the smoke produced by six different types of cigarette. Rev Mal Respir, 24, 845-852.

Beher, D., Wu, J., Cumine, S., Kim, K. W., Lu, S. C., Atangan, L. and Wang, M. (2009). Resveratrol is not a direct activator of SIRT1 enzyme activity. Chem Biol & Drug Des, 74, 619-624.

Belguendouz, L., Fremont, L. and Linard, A. (1997). Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. Biochem Pharmacol, 53, 1347-1355.

Belitskaya-Levy, I., Hajjou, M., Su, W. C., Yie, T. A., Tchou-Wong, K. M., Tang, M. S., Goldberg, J. D. and Rom, W. N. (2007). Gene profiling of normal human bronchial epithelial cells in response to asbestos and benzo(a)pyrene diol epoxide (BPDE). J Environ Pathol Toxicol Oncol, 26, 281-294.

Bellien, J., Thuillez, C. and Joannides, R. (2008). Contribution of endothelium-derived hyperpolarizing factors to the regulation of vascular tone in humans. Fundam Clin Pharmacol, 22, 363-377.

Benditt, E. P. and Benditt, J. M. (1973). Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci U S A, 70, 1753-1756.

Benowitz, N. L. and Gourlay, S. G. (1997). Cardiovascular toxicity of nicotine: implications for nicotine replacement therapy. J Am Coll Cardiol, 29, 1422-1431.

Berge, G., Ovrebo, S., Eilertsen, E., Haugen, A. and Mollerup, S. (2004). Analysis of resveratrol as a lung cancer chemopreventive agent in A//J mice exposed to benzo[a]pyrene. Br J Cancer, 91, 1380-1383.

Berglund, G. and Wilhelmsen, L. (1975). Factors related to blood pressure in a general population sample of Swedish men. Acta Med Scand, 198, 291-298.

Bernstein, D. M. (2004). A review of the influence of particle size, puff volume, and inhalation pattern on the deposition of cigarette smoke particles in the respiratory tract. Inhal Toxicol, 16, 675-689.

Bhalla, D. K. (1999). Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. J Toxicol and Environ Health B Crit Rev, 2, 31 - 86.

Bhalla, D. K., Hirata, F., Rishi, A. K. and Gairola, C. G. (2009). Cigarette smoke, inflammation, and lung injury: a mechanistic perspective. J Toxicol Environ Health B Crit Rev, 12, 45-64.

Binková, B., Smerhovský, Z., Strejc, P., Boubelík, O., Stávková, Z., Chvátalová, I. and Srám, R. J. (2002). DNA-adducts and atherosclerosis: a study of accidental and sudden death males in the Czech Republic. Mut Res Fund Mol Mech Mut, 501, 115-128.

Borgerding, M. and Klus, H. (2005). Analysis of complex mixtures - Cigarette smoke. Exp Toxicol Pathol, 57, 43-73.

Borland, C., Harmes, K., Cracknell, N., Mack, D. and Higenbottam, T. (1985). Methemoglobin levels in smokers and non-smokers. Arch Environ Health, 40, 330-333.

Borlaug, B. A., Lam, C. S., Roger, V. L., Rodeheffer, R. J. and Redfield, M. M. (2009). Contractility and ventricular systolic stiffening in hypertensive heart disease insights into the pathogenesis of heart failure with preserved ejection fraction. J Am Coll Cardiol, 54, 410-418.

Borra, M. T., Smith, B. C. and Denu, J. M. (2005). Mechanism of Human SIRT1 Activation by Resveratrol. J Biol Chem, 280, 17187-17195.

Brunnberg, S., Andersson, P., Lindstam, M., Paulson, I., Poellinger, L. and Hanberg, A. (2006). The constitutively active Ah receptor (CA-AhR) mouse as a potential model for dioxin exposure--Effects in vital organs. Toxicology, 224, 191-201.

Bugiak, B. and Weber, L. P. (2009). Hepatic and vascular mRNA expression in adult zebrafish (Danio rerio) following exposure to benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Aquat Toxicol, 95, 299-306.

Burns, J., Yokota, T., Ashihara, H., Lean, M. E. J. and Crozier, A. (2002). Plant foods and herbal sources of resveratrol. J Agric Food Chem, 50, 3337-3340.

Burstyn, I. Kromhout, H. Partanen, T. Svane, O. Langard, S. Ahrens, W. Kauppinen, T. Stucker, I. Shaham, J. Heederik, D. Ferro, G. Heikkila, P. Hooiveld, M. Johansen, C. Randem, B. G. Boffetta, P. (2005). Polycyclic aromatic hydrocarbons and fatal ischemic heart disease. Epidemiology, 16, 744-750

Busse, R. and Fleming, I. (2006). Vascular endothelium and blood flow. Handb Exp Pharmacol, 43-78.

Canistro, D., Bonamassa, B., Pozzetti, L., Sapone, A., Abdel-Rahman, S. Z., Biagi, G. L. and Paolini, M. (2009). Alteration of xenobiotic metabolizing enzymes by resveratrol in liver and lung of CD1 mice. Food Chem Toxicol, 47, 454-461.

Carluccio, M. A., Siculella, L., Ancora, M. A., Massaro, M., Scoditti, E., Storelli, C., Visioli, F., Distante, A. and De Caterina, R. (2003). Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol, 23, 622-629.

Carpagnano, G. E., Kharitonov, S. A., Foschino-Barbaro, M. P., Resta, O., Gramiccioni, E. and Barnes, P. J. (2003). Increased inflammatory markers in the exhaled breath condensate of cigarette smokers. Eur Respir J, 21, 589-593.

Casper, R. F., Quesne, M., Rogers, I. M., Shirota, T., Jolivet, A., Milgrom, E. and Savouret, J. F. (1999). Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. Mol Pharmacol, 56, 784-790.

Castillo, M. J., Nakajima, K., Zimmerman, M. and Powers, J. C. (1979). Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. Anal Biochem, 99, 53-64.

Celermajer, D. S., Adams, M. R., Clarkson, P., Robinson, J., McCredie, R., Donald, A. and Deanfield, J. E. (1996). Passive smoking and impaired endothelium-dependent arterial dilatation in healthy young adults. N Engl J Med, 334, 150-155.

Center for Disease Control and prevention. (2003). Chartbook on Trends in the Health of Americans., N. C. f. H. Statistics;, Ed, Hyattsville, MD, .

Center for Disease Control and Prevention. (2006). The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General, D. o. H. a. H. S. C. o. D. C. a. Prevention, Ed, Atlanta GA.

Chan-Yeung, M. and Dimich-Ward, H. (2003). Respiratory health effects of exposure to environmental tobacco smoke. Respirology, 8, 131-139.

Charavaryamath, C., Janardhan, K. S., Townsend, H. G., Willson, P. and Singh, B. (2005). Multiple exposures to swine barn air induce lung inflammation and airway hyper-responsiveness. Respir Res, 6, 50.

Chen, C. K. and Pace-Asciak, C. R. (1996). Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. Gen Pharmacol, 27, 363-366.

Churg, A., Cosio, M. and Wright, J. L. (2008). Mechanisms of cigarette smoke-induced COPD: insights from animal models. Am J Physiol Lung Cell Mol Physiol, 294, L612-631.

Churg, A., Wang, R., Wang, X., Onnervik, P.-O., Thim, K. and Wright, J. L. (2007). Effect of an MMP-9/MMP-12 inhibitor on smoke-induced emphysema and airway remodelling in guinea pigs. Thorax, 62:, 706-713.

Churg, A., Wang, R. D., Tai, H., Wang, X., Xie, C., Dai, J., Shapiro, S. D. and Wright, J. L. (2003). Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-{alpha} release. Am. J. Respir. Crit. Care Med., 167, 1083-1089.

Ciolino, H. P., Daschner, P. J. and Yeh, G. C. (1998). Resveratrol inhibits transcription of cyp1a1 in vitro by preventing activation of the aryl hydrocarbon receptor. Canr Res, 58, 5707-5712.

Cohen, H. Y., Miller, C., Bitterman, K. J., Wall, N. R., Hekking, B., Kessler, B., Howitz, K. T., Gorospe, M., de Cabo, R. and Sinclair, D. A. (2004). Calorie restriction promotes mammalian cell survival by inducing the sirt1 deacetylase. Science, 305, 390-392.

Collins, R. G., Velji, R., Guevara, N. V., Hicks, M. J., Chan, L. and Beaudet, A. L. (2000). P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med, 191, 189-194.

Commins, B. T., Cooper, R. L. and Lindsey, A. J. (1954). Polycyclic hydrocarbons in cigarette smoke. Br J Cancer, 8, 296-302.

Creager, M. A., Luscher, T. F., Cosentino, F. and Beckman, J. A. (2003). Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. Circulation, 108, 1527-1532.

Crowther, M. A. (2005). Pathogenesis of atherosclerosis. Hematology, 2005, 436-441.

Csiszar, A., Labinskyy, N., Podlutsky, A., Kaminski, P. M., Wolin, M. S., Zhang, C., Mukhopadhyay, P., Pacher, P., Hu, F., de Cabo, R., Ballabh, P. and Ungvari, Z. (2008). Vasoprotective effects of resveratrol and SIRT1: attenuation of cigarette smoke-induced oxidative stress and proinflammatory phenotypic alterations. Am J Physiol Heart Circ Physiol, 294, H2721-2735.

Dansky, H. M., Barlow, C. B., Lominska, C., Sikes, J. L., Kao, C., Weinsaft, J., Cybulsky, M. I. and Smith, J. D. (2001). Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. Arterioscler Thromb Vasc Biol, 21, 1662-1667.

de la Lastra, C. A. and Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochem Soc Trans, 35, 1156-1160.

Dinarello, C. A. (2000). Proinflammatory Cytokines. Chest, 118, 503-508.

Ding, Y. S., Zhang, L., Jain, R. B., Jain, N., Wang, R. Y., Ashley, D. L. and Watson, C. H. (2008). levels of tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons in mainstream smoke from different tobacco varieties. Cancer Epidemiol Biomarkers Prev, 17, 3366-3371.

Donnelly, L. E., Newton, R., Kennedy, G. E., Fenwick, P. S., Leung, R. H. F., Ito, K., Russell, R. E. K. and Barnes, P. J. (2004). Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. Am J Physiol Lung Cell Mol Physiol, 287, L774-783.

Duncan, B. B., Schmidt, M. I. s., Pankow, J. S., Ballantyne, C. M., Couper, D., Vigo, A., Hoogeveen, R., Folsom, A. R. and Heiss, G. (2003). Low-grade systemic inflammation and the development of type 2 diabetes. Diabetes, 52, 1799-1805.

Eatough DJ, H. L., Lewis EA. . (1990). The chemical characterisation of environmental tobacco smoke. Proceedings of the International Symposium at Mcgill University Massachusetts, pp. 3-39.

Ector, B. J., Magee, J. B., Hegwood, C. P. and Coign, M. J. (1996). Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds, and wines. Am. J. Enol. Vitic., 47, 57-62.

Eileen, L. T. (2006). Lung inflammatory responses. Vet. Res., 37, 469-486.

Eisner, M., Balmes, J., Yelin, E., Katz, P., Hammond, S. K., Benowitz, N. and Blanc, P. (2006). Directly measured secondhand smoke exposure and COPD health outcomes. BMC Pulmonary Medicine, 6, 12.

Eisner, M. D., Jacob, P., III, Benowitz, N. L., Balmes, J. and Blanc, P. D. (2009). Longer term exposure to secondhand smoke and health outcomes in COPD: Impact of urine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Nicotine Tob Res, 11, 945-953.

ElAttar, T. M. and Virji, A. S. (1999). Modulating effect of resveratrol and quercetin on oral cancer cell growth and proliferation. Anticancer Drugs, 10, 187-193.

Endemann, D. H. and Schiffrin, E. L. (2004). Endothelial Dysfunction. J Am Soc Nephrol, 15, 1983-1992.

Fang, Q., Sun, H. and Mayhan, W. G. (2003). Impairment of nitric oxide synthase-dependent dilatation of cerebral arterioles during infusion of nicotine. Am J Physiol Heart Circ Physiol, 284, H528-534.

Fujii, T., Hayashi, S., Hogg, J. C., Mukae, H., Suwa, T., Goto, Y., Vincent, R. and van Eeden, S. F. (2002). Interaction of alveolar macrophages and airway epithelial cells following

exposure to particulate matter produces mediators that stimulate the bone marrow. Am. J. Respir. Cell Mol. Biol., 27, 34-41.

Fukao, H., Ijiri, Y., Miura, M., Hashimoto, M., Yamashita, T., Fukunaga, C., Oiwa, K., Kawai, Y., Suwa, M. and Yamamoto, J. (2004). Effect of trans-resveratrol on the thrombogenicity and atherogenicity in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. Blood Coagul Fibrinolysis, 15, 441-446.

Galkina, E. and Ley, K. (2007). vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol, 27, 2292-2301.

Gan, W. Q., Man, S. F. P. and Sin, D. D. (2005). The interactions between cigarette smoking and reduced lung function on systemic inflammation. Chest, 127, 558-564.

Gentner, N. J. and Weber, L.P. (2010a). Arterial stiffness and altered circadian blood pressure patterns from environmental tobacco smoke exposure in rats is mediated by lung inflammation and oxidative stress. Submitted to the Journal of Hypertension.

Gentner, N. J. and Weber, L.P. (2010b). Intranasal benzo[a]pyrene alters circadian blood pressure patterns and causes lung inflammation in rats. Archives of Toxicology *doi:10.1007/s00204-010-0589-6*.

Gentner, N. J. and Weber, L.P. (2010c). Using blood pressure telemetry to assess acute changes in arterial stiffness in rats after nitric oxide synthase inhibition or environmental tobacco smoke exposure. Can J Physiol Pharmacol, 88, 918-928.

Godschalk, R., Curfs, D. l., Bartsch, H., Van Schooten, F.-J. and Nair, J. (2003). Benzo[a]pyrene enhances lipid peroxidation induced dna damage in aorta of apolipoprotein e knockout mice. Free Radic Res, 37, 1299 - 1305.

Goldstein, J. L. and Brown, M. S. (1977). The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem, 46, 897-930.

Green, D. (2005). Point: Flow-mediated dilation does reflect nitric oxide-mediated endothelial function. J Appl Physiol, 99, 1233-1234; discussion 1237-1238.

Guatura, S. B., Martinez, J. A., Santos Bueno, P. C. and Santos, M. L. (2000). Increased exhalation of hydrogen peroxide in healthy subjects following cigarette consumption. Sao Paulo Med J, 118, 93-98.

Gulel, O., Soylu, K., Yazici, M., Demircan, S., Durna, K. and Sahin, M. (2007). Longitudinal diastolic myocardial functions are affected by chronic smoking in young healthy people: a study of color tissue Doppler imaging. Echocardiography, 24, 494-498.

Haider, U. G. B., Sorescu, D., Griendling, K. K., Vollmar, A. M. and Dirsch, V. M. (2003). Resveratrol increases serine15-phosphorylated but transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells. Mol Pharmacol, 63, 925-932.

Hamasaki, H., Sato, J., Masuda, H., Tamaoki, S., Isotani, E., Obayashi, S., Udagawa, T. and Azuma, H. (1997). Effect of nicotine on the intimal hyperplasia after endothelial removal of the rabbit carotid artery. Gen Pharmacol Vascular Sys, 28, 653-659.

Han, Z., Miwa, Y., Obikane, H., Mitsumata, M., Takahashi-Yanaga, F., Morimoto, S. and Sasaguri, T. (2008). Aryl hydrocarbon receptor mediates laminar fluid shear stress-induced CYP1A1 activation and cell cycle arrest in vascular endothelial cells. Cardiovasc Res, 77, 809-818.

Hanyu, O., Yoshida, J., Abe, E., Hirayama, S., Miyake, K., Aizawa, Y. and Miida, T. (2009). High-sensitivity CRP reflects insulin resistance in smokers. J Atheroscler Thromb, 16, 560-567.

Haritash, A. K. and Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. J Haz Mat, 169, 1-15.

Harris, R. A., Nishiyama, S. K., Wray, D. W. and Richardson, R. S. (2010). Ultrasound assessment of flow-mediated dilation. Hypertension, 55, 1075-1085.

Hasenkam, J. M., Ostergaard, J. H., Pedersen, E. M., Paulsen, P. K., Nygaard, H., Schurizek, B. A. and Johannsen, G. (1988). A model for acute haemodynamic studies in the ascending aorta in pigs. Cardiovasc Res, 22, 464-471.

Hecht, S. S. (1999). Tobacco Smoke Carcinogens and Lung Cancer. J. Natl. Cancer Inst., 91, 1194-1210.

Hein, T. W., Singh, U., Vasquez-Vivar, J., Devaraj, S., Kuo, L. and Jialal, I. (2009). Human induces endothelial dysfunction and uncoupling of eNOS in vivo. Atherosclerosis, 206, 61-68.

Heiss, C., Sievers, R. E., Amabile, N., Momma, T. Y., Chen, Q., Natarajan, S., Yeghiazarians, Y. and Springer, M. L. (2008). In vivo measurement of flow-mediated vasodilation in living rats using high-resolution ultrasound. Am J Physiol Heart Circ Physiol, 294, H1086-1093.

Hellermann, G., Nagy, S., Kong, X., Lockey, R. and Mohapatra, S. (2002). Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. Resp Res, 3, 22.

Higgins, M. (1991). Risk factors associated with chronic obstructive lung disease. Ann N Y Acad Sci, 624, 7-17.

Hoffmann, D. and Hoffmann, I. (1997). The changing cigarette, 1950-1995. J Toxicol Environ Health, 50, 307 - 364.

Hoffmann, D., Hoffmann, I. and El-Bayoumy, K. (2001). The less harmful cigarette: a controversial issue. a tribute to Ernst L. Wynder. Chem Res Toxicol, 14, 767-790.

Huang, A., Sun, D., Carroll, M. A., Jiang, H., Smith, C. J., Connetta, J. A., Falck, J. R., Shesely, E. G., Koller, A. and Kaley, G. (2001). EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS-KO mice. Am J Physiol Heart Circ Physiol, 280, H2462-2469.

Ilebekk, A. and Lekven, J. (1974). Cardiac effects of nicotine in dogs. Scand J Clin Lab Invest, 33, 153-159.

Ischiropoulos, H. and al-Mehdi, A. B. (1995). Peroxynitrite-mediated oxidative protein modifications. FEBS Lett, 364, 279-282.

Izzotti, A., De Flora, S., Petrilli, G. L., Gallagher, J., Rojas, M., Alexandrov, K., Bartsch, H. and Lewtas, J. (1995). Cancer biomarkers in human atherosclerotic lesions: detection of DNA adducts. Can Epi Biom Prevn, 4, 105-110.

Jacobsen, L. K., Krystal, J. H., Mencl, W. E., Westerveld, M., Frost, S. J. and Pugh, K. R. (2005). Effects of smoking and smoking abstinence on cognition in adolescent tobacco smokers. Bioll Psych, 57, 56-66.

Jaimes, E. A., DeMaster, E. G., Tian, R.-X. and Raij, L. (2004). Stable Compounds of Cigarette Smoke Induce Endothelial Superoxide Anion Production via NADPH Oxidase Activation. Arterioscler Thromb Vasc Biol, 24, 1031-1036.

Janoff, A., Raju, L. and Dearing, R. (1983). Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. Am Rev Respir Dis, 127, 540-544.

Jin, A. S., Hyunah, L., Yoo-Kyoung, L., Yaekyu, K., Ji Ha, C. and Eun-Mi, P. (2010). Therapeutic effects of resveratrol during acute periods following experimental ischemic stroke. J neuroim, 227, 93-100.

Kallio, K., Jokinen, E., Raitakari, O. T., Hamalainen, M., Siltala, M., Volanen, I., Kaitosaari, T., Viikari, J., Ronnemaa, T. and Simell, O. (2007). Tobacco smoke exposure is associated with attenuated endothelial function in 11-year-old healthy children. Circulation, 115, 3205-3212.

Kao, Y. H., Mohler, E. R., Arger, P. H. and Sehgal, C. M. (2003). Brachial artery: measurement of flow-mediated dilatation with cross-sectional us technical validation1. Radiology, 228, 895-900.

Karatzi, K., Papamichael, C., Karatzis, E., Papaioannou, T. G., Voidonikola, P. T., Lekakis, J. and Zampelas, A. (2007). Acute smoking induces endothelial dysfunction in healthy smokers. is this reversible by red wine's antioxidant constituents? J Am Coll Nutr, 26, 10-15.

Kawada, N., Seki, S., Inoue, M. and Kuroki, T. (1998). Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology, 27, 1265-1274.

Kilburn, K. H. and McKenzie, W. (1975). Leukocyte recruitment to airways by cigarette smoke and particle phase in contrast to cytotoxicity of vapor. Science, 189, 634-637.

Knaapen, A. M., Curfs, D. M., Pachen, D. M., Gottschalk, R. W., de Winther, M. P., Daemen, M. J. and Van Schooten, F. J. (2007). The environmental carcinogen benzo[a]pyrene

induces expression of monocyte-chemoattractant protein-1 in vascular tissue: a possible role in atherogenesis. Mutat Res, 621, 31-41.

Kobayashi, J., Kihira, Y. and Kitamura, S. (1988). Effects of cigarette smoking on blood levels of leukotrienes and plasma levels of complements C3a and C5a in healthy volunteers. Arch Env Health Inter J, 43, 371 - 374.

Kobayashi, S., Okamoto, H., Iwamoto, T., Toyama, Y., Tomatsu, T., Yamanaka, H. and Momohara, S. (2008). A role for the aryl hydrocarbon receptor and the dioxin TCDD in rheumatoid arthritis. Rheumatology, 47, 1317-1322.

Kode, A., Rajendrasozhan, S., Caito, S., Yang, S.-R., Megson, I. L. and Rahman, I. (2008). Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. Am J Physiol Lung Cell Mol Physiol, 294, L478-488.

Kopf, P. G. and Walker, M. K. (2010) 2,3,7,8-tetrachlorodibenzo-p-dioxin increases reactive oxygen species production in human endothelial cells via induction of cytochrome P4501A1. Toxicol Appl Pharmacol, 245, 91-99.

Korashy, H. M. and El-Kadi, A. O. S. (2006). The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases. Drug Met Rev, 38, 411 - 450.

Krupski, W. C. (1991). The peripheral vascular consequences of smoking. Ann Vasc Surg, 5, 291-304.

Kubo, S., Kobayashi, M., Masunaga, Y., Ishii, H., Hirano, Y., Takahashi, K. and Shimizu, Y. (2005). Cytokine and chemokine expression in cigarette smoke-induced lung injury in guinea pigs. Eur Resp J, 26, 993-1001.

Kumar V, A. A., Fausto N, Aster J. (2010). Robbins & Cotran Pathologic Basis of Disease Saunders-Elsevie, Philadelphia.

Kunitomo, M., Yamaguchi, Y., Kagota, S., Yoshikawa, N., Nakamura, K. and Shinozuka, K. (2009). Biochemical evidence of atherosclerosis progression mediated by increased oxidative stress in apolipoprotein E-deficient spontaneously hyperlipidemic mice exposed to chronic cigarette smoke. J Pharmacol Sci, 110, 354-361.

Kurtz, T. W., Griffin, K. A., Bidani, A. K., Davisson, R. L. and Hall, J. E. (2005). Recommendations for blood pressure measurement in humans and experimental animals. Part 2: Blood pressure measurement in experimental animals: a statement for professionals from the subcommittee of professional and public education of the American Heart Association council on high blood pressure research. Hypertension, 45, 299-310.

Kuschner, W. G., D'Alessandro, A., Wong, H. and Blanc, P. D. (1996). Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. Eur Resp J, 9, 1989-1994.

Lassegue, B. and Clempus, R. E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. Am J Physiol Regul Integr Comp Physiol, 285, R277-297.

Lekli, I., Szabo, G., Juhasz, B., Das, S., Das, M., Varga, E., Szendrei, L., Gesztelyi, R., Varadi, J., Bak, I., Das, D. K. and Tosaki, A. (2008). Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin. Am J Physiol Heart Circ Physiol, 294, H859-866.

Levin, W., Wood, A., Chang, R., Ryan, D., Thomas, P., Yagi, H., Thakker, D., Vyas, K., Boyd, C., Chu, S.-Y., Conney, A. and Jerina, D. (1982). Oxidative Metabolism of Polycyclic Aromatic Hydrocarbons to Ultimate Carcinogens. Drug Met Rev 13, 555 - 580.

Li, D. and Mehta, J. L. (2005). Oxidized LDL, a critical factor in atherogenesis. Cardiovas Res, 68, 353-354.

Libby, P. (2002). Inflammation in atherosclerosis. Nature, 420, 868-874.

Lin, S.-J., Defossez, P.-A. and Guarente, L. (2000). Requirement of NAD and SIR2 for Life-Span Extension by Calorie Restriction in Saccharomyces cerevisiae. Science, 289, 2126-2128.

Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., De Simone, G., Ferguson, T. B., Ford, E., Furie, K., Gillespie, C., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, P. M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M. M., Meigs, J., Mozaffarian, D., Mussolino, M., Nichol, G., Roger, V. L., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Thom, T., Wasserthiel-Smoller, S., Wong, N. D. and Wylie-Rosett, J. Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation, 121, 948-954.

Lodovici, M., Akpan, V., Evangelisti, C. and Dolara, P. (2004). Sidestream tobacco smoke as the main predictor of exposure to polycyclic aromatic hydrocarbons. J Appl Toxicol, 24, 277-281.

Lucas, A. D. and Greaves, D. R. (2001). Atherosclerosis: role of chemokines and macrophages. Expert Rev Mol Med, 3, 1-18.

Lucattelli, M., Bartalesi, B., Cavarra, E., Fineschi, S., Lunghi, B., Martorana, P. A. and Lungarella, G. (2005). Is neutrophil elastase the missing link between emphysema and fibrosis? Evidence from two mouse models. Respir Res, 6, 83.

Luscher, T. F. (1994). The endothelium and cardiovascular disease -- a complex relation. N Engl J Med, 330, 1081-1083.

MacNee, W., Maclay, J. and McAllister, D. (2008). Cardiovascular injury and repair in chronic obstructive pulmonary disease. Proc Am Thorac Soc, 5, 824-833.

Maeno, T., Houghton, A. M., Quintero, P. A., Grumelli, S., Owen, C. A. and Shapiro, S. D. (2007). CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. J Immunol, 178, 8090-8096.

Mahonen, M. S., McElduff, P., Dobson, A. J., Kuulasmaa, K. A. and Evans, A. E. (2004). Current smoking and the risk of non-fatal myocardial infarction in the WHO MONICA Project populations. Tobacco Control, 13:, 244-250.

Makomaski Illing, E. M. and Kaiserman, M. J. (2004). Mortality attributable to tobacco use in Canada and its regions, 1998. Can J Public Health, 95, 38-44.

Malerba, M. and Romanelli, G. (2009). Early cardiovascular involvement in chronic obstructive pulmonary disease. Monaldi Arch Chest Dis, 71, 59-65.

Martin, S. (2009). Flow-mediated dilatation: a review of techniques and applications. Echocardiography, 26, 832-841.

May, A. and Wang, T. J. (2007). Evaluating the role of biomarkers for cardiovascular risk prediction: focus on CRP, BNP and urinary microalbumin. Expert Rev Mol Diagn, 7, 793-804.

Mehta, J. L., Rasouli, N., Sinha, A. K. and Molavi, B. (2006). Oxidative stress in diabetes: A mechanistic overview of its effects on atherogenesis and myocardial dysfunction. Int J Biochem Cell Biol, 38, 794-803.

Meuronen, A., Majuri, M. L., Alenius, H., Mantyla T., Wolff, H., Piirila, P. and Laitinen, A. (2008). Decreased cytokine and chemokine mrna expression in bronchoalveolar lavage in asymptomatic smoking subjects. Respiration, 75, 450-458.

Meurrens, K., Ruf, S., Ross, G., Schleef, R., von Holt, K. and Schlater, K. D. (2007). Smoking accelerates the progression of hypertension-induced myocardial hypertrophy to heart failure in spontaneously hypertensive rats. Cardiovas Res, 76, 311-322.

Miller, Y. I., Choi, S. H., Fang, L. and Tsimikas, S. (2010). Lipoprotein modification and macrophage uptake: role of pathologic cholesterol transport in atherogenesis. Subcell Biochem, 51, 229-251.

Mills, P. R., Davies, R. J. and Devalia, J. L. (1999). Airway epithelial cells, cytokines, and pollutants. Am. J. Respir. Crit. Care Med., 160, S38-43.

Minicucci, M. F., Azevedo, P. S., Paiva, S. A. and Zornoff, L. A. (2009). Cardiovascular remodeling induced by passive smoking. Inflamm Allergy Drug Targets, 8, 334-339.

Mokdad, A. H., Marks, J. S., Stroup, D. F. and Gerberding, J. L. (2004). Actual causes of death in the united states, 2000. JAMA, 291, 1238-1245.

Montuschi, P., Collins, J. V., Ciabattoni, G., Lazzeri, N., Corradi, M., Kharitonov, S. A. and Barnes, P. J. (2000). Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with copd and healthy smokers. Am. J. Respir. Crit. Care Med., 162, 1175-1177.

Moorthy, B., Miller, K. P., Jiang, W., Williams, E. S., Kondraganti, S. R. and Ramos, K. S. (2003). Role of cytochrome P4501B1 in benzo[a]pyrene bioactivation to DNA-binding metabolites in mouse vascular smooth muscle cells: evidence from 32P-postlabeling for formation of 3-hydroxybenzo[a]pyrene and benzo[a]pyrene-3,6-quinone as major proximate genotoxic intermediates. J Pharmacol Exp Ther, 305, 394-401.

Morrison, D., Rahman, I., Lannan, S. and Macnee, W. (1999). Epithelial permeability, inflammation, and oxidant stress in the air spaces of smokers. Am. J. Respir. Crit. Care Med., 159, 473-479.

Mullen, M. J., Kharbanda, R. K., Cross, J., Donald, A. E., Taylor, M., Vallance, P., Deanfield, J. E. and MacAllister, R. J. (2001). Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo : relevance to endothelial dysfunction in hypercholesterolemia. Circ Res, 88, 145-151.

Munzel, T., Daiber, A., Ullrich, V. and Mulsch, A. (2005). Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cgmp-dependent protein kinase. Arterioscler Thromb Vasc Biol, 25, 1551-1557.

N'Diaye, M., Le Ferrec, E., Lagadic-Gossmann, D., Corre, S. b., Gilot, D., Lecureur, V. r., Monteiro, P., Rauch, C., Galibert, M.-D. and Fardel, O. (2006). Aryl hydrocarbon receptor- and calcium-dependent induction of the chemokine ccl1 by the environmental contaminant benzo[a]pyrene. J Biol Chem, 281, 19906-19915.

Nagai, K., Betsuyaku, T., Kondo, T., Nasuhara, Y. and Nishimura, M. (2006). Long term smoking with age builds up excessive oxidative stress in bronchoalveolar lavage fluid. Thorax, 61:, 496-502.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y. and Dalton, T. P. (2000). Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem Pharmacol, 59, 65-85.

O'Donnell, C. J. and Kannel, W. B. (1998). Cardiovascular risks of hypertension: lessons from observational studies. J Hypertens Suppl, 16, S3-7.

Ockene, I. S. and Miller, N. H. (1997). Cigarette smoking, cardiovascular disease, and stroke : a statement for healthcare professionals from the american heart association. Circulation, 96, 3243-3247.

Oesterling, E., Toborek, M. and Hennig, B. (2008). Benzo[a]pyrene induces intercellular adhesion molecule-1 through a caveolae and aryl hydrocarbon receptor mediated pathway. Toxicol App Pharmacol, 232, 309-316.

Orallo, F., Alvarez, E., Camiaa, M., Leiro, J. M., Gamez, E. and Fernandez, P. (2002). The possible implication of trans-resveratrol in the cardioprotective effects of long-term moderate wine consumption. Mol Pharmacol, 61, 294-302.

Orozco, L. D., Kapturczak, M. H., Barajas, B., Wang, X., Weinstein, M. M., Wong, J., Deshane, J., Bolisetty, S., Shaposhnik, Z., Shih, D. M., Agarwal, A., Lusis, A. J. and Araujo, J. A. (2007). Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. Circ Res, 100, 1703-1711.

Osterud, B. and Bjorklid, E. (2003). Role of monocytes in atherogenesis. Physiol. Rev., 83, 1069-1112.

Pacholec, M., Bleasdale, J. E., Chrunyk, B., Cunningham, D., Flynn, D., Garofalo, R. S., Griffith, D., Griffor, M., Loulakis, P., Pabst, B., Qiu, X., Stockman, B., Thanabal, V., Varghese, A., Ward, J., Withka, J. and Ahn, K. (2010). SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J Biol Chem, 285, 8340-8351.

Packard, R. R. S. and Libby, P. (2008). Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem, 54, 24-38.

Palsamy, P., Sivakumar, S. and Subramanian, S. Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in

streptozotocin-nicotinamide-induced experimental diabetic rats. Chem Biol Interact, 186, 200-210.

Paule, W. J., Zemplenyi, T. K., Rounds, D. E. and Blankenhorn, D. H. (1976). Light- and electron-microscopic characteristics of arterial smooth muscle cell cultures subjected to hypoxia or carbon monoxide. Atherosclerosis, 25, 111-123.

Pauly, John, L., Allison, Erin, M., Hurley, Edward, L., Nwogu, Chukwumere, E., Wallace, Paul, K., Paszkiewicz and Geraldine, M. (2005). Fluorescent human lung macrophages analyzed by spectral confocal laser scanning microscopy and multispectral cytometry. Wiley-Liss, New York, NY, ETATS-UNIS, pp. 11.

Pei, X.-H., Nakanishi, Y., Inoue, H., Takayama, K., Bai, F. and Hara, N. (2002). Polycyclic aromatic hydrocarbons induce il-8 expression through nuclear factor [kappa]b activation in a549 cell line. Cytokine, 19, 236-241.

Peinado, Victor I., Barbera, Joan A., Abate, P., Ramirez, J., Roca, J., Santos, S. and Rodriguez-Roisin, R. (1999). Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med., 159, 1605-1611.

Peluffo, G., Calcerrada, P., Piacenza, L., Pizzano, N. and Radi, R. (2009a). Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular endothelium: studies in cultured cells and smokers. Am J Physiol Heart Circ Physiol, 296, H1781-1792.

Peluffo, G., Calcerrada, P., Piacenza, L., Pizzano, N. and Radi, R. (2009b). Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular

endothelium: studies in cultured cells and smokers. Am J Physiol Heart Circ Physiol, 296, H1781-1792.

Penn, A. and Snyder, C. (1988). Arteriosclerotic plaque development is 'promoted' by polynuclear aromatic hydrocarbons. Carcinogenesis, 9, 2185-2189.

Penumathsa, S., Koneru, S., Samuel, S. M., Zhan, L., Menon, V. P. and Maulik, N. (2008). Red wine polyphenol resveratrol regulates Heme Oxygenase-1 conversely for the disruption of Caveolin-1/eNOS platform and improve left ventricular function in Ischemic Hypercholesterolemic Myocardium. FASEB J., 22, 1130.1113-.

Pickering, T. G., Hall, J. E., Appel, L. J., Falkner, B. E., Graves, J., Hill, M. N., Jones, D. W., Kurtz, T., Sheps, S. G. and Roccella, E. J. (2005). Recommendations for blood pressure measurement in humans and experimental animals: part 1: blood pressure measurement in humans: a statement for professionals from the subcommittee of professional and public education of the american heart association council on high blood pressure research. Hypertension, 45, 142-161.

Pinto-Plata, V. M., Mallerova, H., Toso, J. F., Feudjo-Tepie, M., Soriano, J. B., Vessey, R. S. and Celli, B. R. (2006). in patients with COPD, control smokers and non-smokers. Thorax, 61, 23-28.

Pittilo, R. M., Bull, H. A., Gulati, S., Rowles, P. M., Blow, C. M., Machin, S. J. and Woolf, N. (1990). Nicotine and cigarette smoking: effects on the ultrastructure of aortic endothelium. Int J Exp Pathol, 71, 573-586.

Podechard, N., Lecureur, V., Le Ferrec, E., Guenon, I., Sparfel, L., Gilot, D., Gordon, J. R., Lagente, V. and Fardel, O. (2008). Interleukin-8 induction by the environmental

contaminant benzo(a)pyrene is aryl hydrocarbon receptor-dependent and leads to lung inflammation. Tox Let, 177, 130-137.

Primatesta, P., Falaschetti, E., Gupta, S., Marmot, M. G. and Poulter, N. R. (2001). Association between smoking and blood pressure : evidence from the health survey for england. Hypertension, 37, 187-193.

Puglia, G. D., Freeman, L. M., Rush, J. E., King, R. G. P. and Crawford, S. L. (2006). Use of a flow-mediated vasodilation technique to assess endothelial function in dogs. American Journal of Veterinary Research, 67, 1533-1540.

Radomski, M. W., Palmer, R. M. and Moncada, S. (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci U S A, 87, 5193-5197.

Rahman, M. M. and Laher, I. (2007). Structural and functional alteration of blood vessels caused by cigarette smoking: an overview of molecular mechanisms. Current Vascular Pharmacology, 5, 276-292.

Renaud, S. and de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet, 339, 1523-1526.

Rocha, K. K., Souza, G. A., Ebaid, G. X., Seiva, F. R., Cataneo, A. C. and Novelli, E. L. (2009). Resveratrol toxicity: effects on risk factors for atherosclerosis and hepatic oxidative stress in standard and high-fat diets. Food Chem Toxicol, 47, 1362-1367.

Ross, R. and Glomset, J. A. (1973). Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science, 180, 1332-1339.

Ryder, M. I., Saghizadeh, M., Ding, Y., Nguyen, N. and Soskolne, A. (2002). Effects of tobacco smoke on the secretion of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta from peripheral blood mononuclear cells. Oral Microbiol Immunol, 17, 331-336.

Ryter, S. W., Alam, J. and Choi, A. M. K. (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol. Rev., 86, 583-650.

Rzucidlo, E. M. (2009). Signaling pathways regulating vascular smooth muscle cell differentiation. Vascular, 17 Suppl 1, S15-20.

Salvi, S. S. and Barnes, P. J. (2009). Chronic obstructive pulmonary disease in non-smokers. Lancet, 374, 733-743.

Sarfaraz, S. and Rohit, A. (2009). Resveratrol: biologic and therapeutic implications. Journal of the Cardiometabolic Syndrome, 4, 102-106.

Sasco, A. J., Secretan, M. B. and Straif, K. (2004). Tobacco smoking and cancer: a brief review of recent epidemiological evidence. Lung Cancer, 45 Suppl 2, S3-9.

Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T. and Masaki, T. (1997). An endothelial receptor for oxidized low-density lipoprotein. Nature, 386, 73-77.

Schick, S. and Glantz, S. A. (2006). Sidestream cigarette smoke toxicity increases with aging and exposure duration. Tobacco Control, 15:, 424-429.

Schulz, E., Jansen, T., Wenzel, P., Daiber, A. and Munzel, T. (2008). Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension. Antioxid Redox Signal, 10, 1115-1126.

Scott, D. A. and Palmer, R. M. (2003). The influence of tobacco smoking on adhesion molecule profiles. Tobacco Induced Diseases, 1, 7 - 25.

Selman, M. s. (2003). The spectrum of smoking-related interstitial lung disorders. Chest, 124, 1185-1187.

Sharafkhaneh, A., Hanania, N. A. and Kim, V. (2008). Pathogenesis of emphysema: from the bench to the bedside. Proc Am Thorac Soc, 5, 475-477.

Shinton, R. and Beevers, G. (1989). Meta-analysis of relation between cigarette smoking and stroke. BMJ, 298, 789-794.

Shopland, D. R., Eyre, H. J. and Peachacek, T. F. (1991). Smoking-attributable cancer mortality in 1991: is lung cancer now the leading cause of death among smokers in the united states? J. Natl. Cancer Inst., 83, 1142-1148.

Silvia, B., Elisabet, A., Gemma, F., Mireia, O., Viktoria, R., Josep, M. A. s. and Francisco, J. L. p.-S. (2007). Resveratrol, a natural diphenol, reduces metastatic growth in an experimental cancer model. Can let, 245, 144-148.

Smith, C. J., Perfetti, T. A., Garg, R. and Hansch, C. (2003). IARC carcinogens reported in cigarette mainstream smoke and their calculated log P values. Food Chem Toxicol, 41, 807-817.

Sørhaug, S., Steinshamn, S., Nilsen, O. G. and Waldum, H. L. (2006). Chronic inhalation of carbon monoxide: Effects on the respiratory and cardiovascular system at doses corresponding to tobacco smoking. Toxicology, 228, 280-290.

Statistics Canada. (2010). Deaths, by cause. Chapter IX: Diseases of the circulatory system (I00 to I99), canada, 2000 to 2006, Ed 1.

Taddei, S., Caraccio, N., Virdis, A., Dardano, A., Versari, D., Ghiadoni, L., Ferrannini, E., Salvetti, A. and Monzani, F. (2006). Low-grade systemic inflammation causes endothelial dysfunction in patients with hashimoto's thyroiditis. J Clin Endocrinol Metab, 91, 5076-5082.

Tamagawa, E., Bai, N., Morimoto, K., Gray, C., Mui, T., Yatera, K., Zhang, X., Xing, L., Li, Y., Laher, I., Sin, D. D., Man, S. F. P. and van Eeden, S. F. (2008). Particulate matter exposure induces persistent lung inflammation and endothelial dysfunction. Am J Physiol Lung Cell Mol Physiol, 295, L79-85.

Tamagawa, E., Suda, K., Yuan, W., Li, X., Mui, T., Yuexin, L., van Eeden, S. F., Man, S. F. P. and Sin, D. D. (2009). Endotoxin-induced translocation of interleukin-6 from lungs to the systemic circulation. Innate Imm, 15, 251-258.

Tanino, M., Betsuyaku, T., Takeyabu, K., Tanino, Y., Yamaguchi, E., Miyamoto, K. and Nishimura, M. (2002). Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. Thorax, 57:, 405-411.

Taybos, G. (2003). Oral Changes Associated with Tobacco Use. Am J Med Sc, 326, 179-182.

Thom, S. R., Xu, Y. A. and Ischiropoulos, H. (1997). Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. Chem Res Toxicol, 10, 1023-1031.

Tithof, P. K., Elgayyar, M., Cho, Y., Guan, W. E. I., Fisher, A. B. and Peters-Golden, M. (2002). Polycyclic aromatic hydrocarbons present in cigarette smoke cause endothelial cell apoptosis by a phospholipase A2-dependent mechanism. FASEB J., 16, 1463-1464.

Turrens, J. F., Lariccia, J. and Nair, M. G. (1997). Resveratrol has no effect on lipoprotein profile and does not prevent peroxidation of serum lipids in normal rats. Free Rad Res, 27, 557-562.

Ueda, A., Ohyanagi, M., Koida, S. and Iwasaki, T. (2005). Enhanced release of endothelium-derived hyperpolarizing factor in small coronary arteries from rats with congestive heart failure. Clin Exp Pharmacol and Physiology, 32, 615-621.

Uno, S., Dalton, T. P., Derkenne, S., Curran, C. P., Miller, M. L., Shertzer, H. G. and Nebert, D. W. (2004). Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome p450 is more important than metabolic activation. Mol Pharmacol, 65, 1225-1237.

van der Vaart, H., Postma, D. S., Timens, W. and Ten Hacken, N. H. T. (2004). Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax, 59, 713-721.

van der Vliet, A., Eiserich, J. P., O'Neill, C. A., Halliwell, B. and Cross, C. E. (1995). Tyrosine modification by reactive nitrogen species: a closer look. Arch Biochem Biophys, 319, 341-349.

Van Lint, P. and Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J Leukoc Biol, 82, 1375-1381.

Van Tiel, E., Peeters, P. H., Smit, H. A., Nagelkerke, N. J., Van Loon, A. J., Grobbee, D. E. and Bueno-de-Mesquita, H. B. (2002). Quitting smoking may restore hematological characteristics within five years. Ann Epidemiol, 12, 378-388.

Vardavas, C. I. and Panagiotakos, D. B. (2009). The causal relationship between passive smoking and inflammation on the development of cardiovascular disease: a review of the evidence. Inflamm Allergy Drug Targets, 8, 328-333.

Verma, A. and Solomon, S. D. (2009). Diastolic dysfunction as a link between hypertension and heart failure. Med Clin North Am, 93, 647-664.

Villablanca, A. C. (1998). Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J Appl Physiol, 84, 2089-2098.

Vogiatzi, G., Tousoulis, D. and Stefanadis, C. (2009). The role of oxidative stress in atherosclerosis. Hellenic J Cardiol, 50, 402-409.

Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., Jr. and Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos, 32, 1377-1382.

Walsh, T., Donnelly, T. and Lyons, D. (2009). Impaired endothelial nitric oxide bioavailability: a common link between aging, hypertension, and atherogenesis? J Am Geriatr Soc, 57, 140-145.

Wang, Z., Zou, J., Cao, K., Hsieh, T. C., Huang, Y. and Wu, J. M. (2005). Dealcoholized red wine containing known amounts of resveratrol suppresses atherosclerosis in hypercholesterolemic rabbits without affecting plasma lipid levels. Int J Mol Med, 16, 533-540.

Weber, L. P., Diamond, S. L., Bandiera, S. M. and Janz, D. M. (2002). Expression of HSP70 and CYP1A protein in ovary and liver of juvenile rainbow trout exposed to beta-naphthoflavone. Comp Biochem Physiol C Toxicol Pharmacol, 131, 387-394.

Whiteley, W., Jackson, C., Lewis, S., Lowe, G., Rumley, A., Sandercock, P., Wardlaw, J., Dennis, M. and Sudlow, C. (2009). Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6. PLoS Med, 6, e1000145.

Wilson, T., Knight, T. J., Beitz, D. C., Lewis, D. S. and Engen, R. L. (1996). Resveratrol promotes atherosclerosis in hypercholesterolemic rabbits. Life Sci, 59, PL15-21.

Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Dansette, P. M., Jerina, D. M. and Conney, A. H. (1976). Mutagenicity and cytotoxicity of benzo(a)pyrene arene oxides, phenols, quinones, and dihydrodiols in bacterial and mammalian cells. Cancer Res, 36, 3350-3357.

Wong, R. H., Howe, P. R., Buckley, J. D., Coates, A. M., Kunz, I. and Berry, N. M. Acute resveratrol supplementation improves flow-mediated dilatation in overweight/obese individuals with mildly elevated blood pressure. Nutr Metab Cardiovasc Dis.

Wright, J. L., Farmer, S. G. and Churg, A. (2002). Synthetic serine elastase inhibitor reduces cigarette smoke-induced emphysema in guinea pigs. Am. J. Respir. Crit. Care Med., 166, 954-960.

Wright, R. O., Lewander, W. J. and Woolf, A. D. (1999). Methemoglobinemia: etiology, pharmacology, and clinical management. Ann Emerg Med, 34, 646-656.

Yach, D. and Wipfli, H. (2006). A century of smoke. Ann Trop Med Parasitol, 100, 465-479.

Yamaguchi, Y., Nasu, F., Harada, A. and Kunitomo, M. (2007). Oxidants in the gas phase of cigarette smoke pass through the lung alveolar wall and raise systemic oxidative stress. J Pharmacol Sci, 103, 275-282.

Yang, L. C., Wang, F. and Liu, M. (1998). A study of an endothelin antagonist from a chinese anti-snake venom medicinal herb. J Cardiovas Pharmacol, 31, S249-S250.

Yang, T., Fu, M., Pestell, R. and Sauve, A. A. (2006). SIRT1 and endocrine signaling. Tre Endoc Met, 17, 186-191.

Yokoyama, M. and Inoue, N. (2004). How vascular NAD(P)H oxidase activity and nox isoform expression are regulated. Arterioscler Thromb Vasc Biol, 24, 1540-1541.

Zhang, W.-Z., Venardos, K., Chin-Dusting, J. and Kaye, D. M. (2006). Adverse effects of cigarette smoke on no bioavailability: role of arginine metabolism and oxidative stress. Hypertension, 48, 278-285.

Zou, M.-H., Leist, M. and Ullrich, V. (1999). Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. Am J Pathol, 154, 1359-1365.

Zou, M.-H., Shi, C. and Cohen, R. A. (2002a). Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. J Clin Invest, 109, 817-826.

Zou, M. H., Shi, C. and Cohen, R. A. (2002b). Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. J Clin Invest, 109, 817-826.

Zou, M. H. and Ullrich, V. (1996). Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. FEBS Letters, 382, 101-104.